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ACCLIMATION OF THE THYROID PROTEINASE
OF THE BURBOT, *LOTA LOTA* LINNAEUS
TO SEASONAL TEMPERATURE CHANGE.

BY

© ALFRED JAMES WIGGS

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Acclimation of the thyroid proteinase of the burbot, Lota Lota Linnaeus to seasonal temperature change" submitted by Alfred James Wiggs, in partial fulfilment for the degree of Doctor of Philosophy.

ABSTRACT

Extracts of thyroid tissue of the burbot *Lota lota* Linnaeus contain at least three proteolytic enzymes with acid pH optima, which can be demonstrated by the use of hemoglobin substrate and the Folin Ciocalteau Phenol reagent method of Anson (1938). The enzymes are characterized by their low temperature stability and their instability to low pH at room temperature. The activity of the enzymes, in crude extracts, is increased by 0.01 M cysteine and inhibited by 0.01 M iodoacetate, glutathione, methyl mercapto-imidazole, ferrous ammonium sulphate, and EDTA, suggesting the presence of both metal-and sulfhydryl-dependent enzymes.

The enzymes appear to function as part of a system responsible for the release of iodoamino acids from thyroglobulin, although they have limited effect on thyroglobulin under the conditions used in this study. Injections of TSH into fish increased the activity of the enzymes which reached a maximum at about six hours after injection at 15 C. Although the burbot was insensitive to the purified TSH preparation at 5 C, they did respond to crude fish pituitary extracts. During the autumn a seasonal change in the sensitivity of the fish thyroid to mammalian TSH was observed. *In vitro* additions of TSH to the enzyme were inhibitory.

The most active enzyme characterized by the lowest optimum pH (enzyme 1), was studied in detail in extracts from fish caught in summer and winter. During the summer the proteinase exhibited an increased resistance

to temperature inactivation and an increased sensitivity to iodoacetate inhibition as well as a higher Michaelis constant. Rate-temperature curves of enzyme 1 showed a change in slope so that Q_{10} values above the critical temperature (temperature at which the slope changed) were generally higher. The critical temperature increased from January to July in step with the increase in environmental temperature.

The distribution of iodide in the burbot is dominated by the skin, which accumulates high concentrations of this ion. Values for skin range from 227 µg% to 816 µg% and serum values range from 7 µg% to 30 µg%. These values indicate the magnitude of seasonal variation which, in the case of serum, appears to be caused by seasonal temperature changes.

The activity of the primary enzyme was greatest in spring and fall when the water temperature was changing, while the relative activities of the enzymes with higher pH optima declined in the winter. These observations are interpreted as indicating that the thyroid in *Lota lota* may be involved in temperature acclimation.

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INTRODUCTION

The importance of the neural and endocrine systems in modifying the direct cellular response which occurs during temperature acclimation of poikilotherms has been the subject of several recent reviews (Precht, 1958, 1964; Prosser, 1962; Jankowsky, 1964). The influence of photoperiod on chill resistance in the goldfish Carassius auratus (Hoar and Robertson, 1959), and on metabolic acclimation in the sunfish Lepomis gibbosus (Roberts, 1964), can be explained satisfactorily only by assuming that some integrating mechanism is involved. Leloup and Fontaine (1960) have shown that temperature acclimation in the eel (Anguilla anguilla) is influenced, in part, by the pituitary. Hoar and Robertson (1959) and Hoar and Eales (1963) feel that photoperiod may act upon the thyroid of the goldfish via the hypothalamo - hypophyseal system to influence temperature acclimation. Hochachka's (1962) work supports this view. In the brook trout (Salvelinus fontinalis) thyroxine treatment produces shifts in glucose metabolism to favor the pentose phosphate pathway (Hochachka, 1962). This pathway becomes important also in fish acclimated to low temperatures (Hochachka and Hayes, 1962).

The research discussed above suggests, but does not prove, a thyroid involvement in temperature acclimation. Several factors have contributed to the difficulty of specifying the thyroid's role. Temperature acclimation is itself a highly complex physiological process (Precht, 1958, 1964; Prosser, 1962; Jankowsky, 1964). Further, the radioisotope methods commonly used to measure thyroid activity in

conjunction with temperature change, have important limitations (Leloup and Fontaine, 1960; Eales, 1964, 1965).

Temperature changes which affect the metabolism of poikilotherms similarly affect the parameters by which thyroid activity is measured. Therefore, it may be difficult to quantify subtle but real changes in thyroid activity (thyroid hormone release) during environmental temperature change. Furthermore, the initial direct metabolic response to an increase or decrease in temperature may be altered subsequently by acclimation. If one can measure the effect of temperature on thyroid activity, it is still difficult to evaluate the physiological significance of the measured change. An example of this difficulty is found in the work of Hoffert and Fromm (1959) who reported that the thyroid hormone secretion rate and total oxygen consumption of rainbow trout (*Salmo gairdnerii*) both decreased with lowered temperatures. These authors concluded that the thyroid had no special role in temperature acclimation. Yet if one calculates from their data the thyroid activity per unit oxygen consumption it is evident that, relative to total oxygen consumption,the thyroid was more active at 3 C than at 13 C.

To correctly interpret the functional significance of changes in thyroid activity during temperature acclimation, one should know the peripheral action and specific function of thyroid hormone. Since this is not possible, it is necessary to relate changes in thyroid activity to some other physiological process such as respiration. Unfortunately, not all tissues respond alike to temperature change. Some tissues, such as brain, compensate for temperature change while others, such as gill, do not (Evans, Purdie, and Hickman, 1962). The oxygen

consumption of various tissues shows a differential response to thyroxine (Barker, 1964) while the concentrations of various enzymes exhibit a variety of responses during temperature acclimation (Prosser, 1962).

In evaluating the effect of the thyroid on temperature acclimation careful consideration must be given to the relative, rather than the absolute, levels of thyroid activity. The results must be viewed in relation to the hormonal requirements of the animal in the new temperature regime and be interpreted in relation to the natural conditions encountered by the animal.

Recently, extensive studies have been made of thyroid enzymes. The activity of these enzymes parallel thyroid activity (Freinkel, 1964). Of those enzymes with a "thyroid specific" function, the thyroid proteinase, first demonstrated by DeRobertis (1941), has received special attention. Studies on the acid proteinase present in the follicular colloid of the thyroid indicated that the enzyme activity reflected changes in thyroid function. Later studies showed that the proteinase responded to thyroid stimulating hormone (TSH) in a variety of animals (Dziemian, 1943; Watson and Trikojus, 1957; Poffenbarger, Powell, and Deiss, 1963; Reinwein, 1964). Subsequent workers showed that the enzyme is probably of lysosomal origin (Wollman, Spicer, and Burstone, 1964; Balasubramiam et al., 1965; Deiss et al., 1966) and that it is probably responsible for the release of thyroid hormone from thyroglobulin (McQuillan et al., 1954).

Wollman et al. (1964) demonstrated that thyroglobulin, as a colloid droplet, is taken into the cell by pinocytosis. The droplet then receives enzymes from lyso-

somes and a localized and controlled hydrolysis occurs within the cell. The proteinase itself is believed to be involved in only the initial stages of hydrolysis (Alpers, Robbins, and Rall, 1955; Alpers, Petermann, and Rall, 1956); the resulting smaller units (polypeptides) are hydrolyzed by a variety of peptidases (Weiss, 1953; Laver and Trikojus, 1956; Jablonsky and McQuillan, 1967; Menzies and McQuillan, 1967). The possibility of synergistic action of the proteinase system with other enzymes (hyaluronidase, α amylase) is recognised (Stoll et al., 1959).

Kress, Peanasky, and Klitgaard (1966) and Dopheide and Trikojus (1966) have purified the thyroid proteinase of the mammal and reported that, as determined by its specificity on the A and B chains of insulin, the enzyme preferentially hydrolyzed bonds involving tyrosine, phenylalanine, leucine, and alanine. Other workers, noticing apparent changes in the properties of the proteinase with purification, have carried out studies which led to the demonstration, in the thyroid, of a wide variety of peptidases and lysosomal acid hydrolases (Llundblad, Bërnback, and Widemann, 1960; Shapland, 1964; Jablonsky and McQuillan, 1967). Recently, Llundblad et al. (1960) have presented evidence that multiple proteinases occur in the human thyroid; this parallels the demonstration of two carboxypeptidases in pig thyroids (Laver and Trikojus, 1956).

While the thyroid proteinase mechanism probably is found in all mammals the nature and distribution of this enzyme in the lower vertebrates has received little attention. Clements and Gorbman (1955) demonstrated a catheptic proteinase in the endostyle of the ammocoetes and concluded that this was a thyroid proteinase. Clements (1957) found that the proteinase from the thyroid of the dogfish (Scyliorhinus canicula) showed different levels of activity in males and females. This

observation agreed with Leloup's (1951) radioisotope evaluation of thyroid activity in this species.

The responsiveness of the thyroid proteinase system to TSH makes it a potentially valuable index of thyroid activity. *In vitro* studies of the extracted enzyme might yield valuable information about possible compensatory responses of the thyroid to temperature change. Such an approach would have the following advantages over radioisotopic methods like hormone secretion rate or radio iodide conversion ratio:

a) Prolonged observations on living fish are not needed.

b) Starvation, stress, and disease during the course of a study are avoided.

c) Thyroid samples can be collected in the field from fish living at different environmental temperatures and the enzyme analyzed at a common temperature in the laboratory. This avoids the many problems associated with having to determine and evaluate thyroid activity with radioiodide, in fish living under uncontrolled environmental conditions.

d) Effects of temperature on certain aspects of thyroid enzyme function can be studied *in vitro* without the complication of thermal acclimation during the study which would occur if intact fish were used.

e) Rate-temperature curves can be determined by assaying enzyme extracts at a series of temperatures, encompassing a temperature range that might be lethal to the intact fish. From these considerations it can be seen that the evaluation of quantitative and qualitative changes in the thyroid proteinase enzyme should provide insight into the mechanism of temperature acclimation in the thyroid gland.

Acclimation of the thyroid may involve direct cellular responses to temp-

erature and indirect effects, acting through the hypothalamus and pituitary gland. There is significant data showing that neural tissue undergoes changes in its cell structure and chemistry (Johnston and Roots, 1964), enzyme levels (Baslow and Nigrelli, 1964), and electrophysiological properties (Konishi and Hickman, 1964) with changes in temperature. Evans et al. (1962) demonstrated that brain respiration in rainbow trout showed rapid and complete acclimation to temperature change and suggest that, in fish, brain acclimation may be a primary compensatory response to temperature. If the hypothalamo-hypophyseal system acclimates as well, compensation at the pituitary level may serve to modify thyroid function when the temperature changes. Since the pituitary in fish, as in mammals, is under hypothalamic (hence central nervous) influence the importance of the pituitary in regulating the activity of the thyroid cannot be overlooked. Changes in pituitary TSH production with increased temperature have been demonstrated in the eel (Leloup and Fontaine, 1960). Nevertheless, acclimation processes within the thyroid gland itself, in response to persistent temperature change, almost certainly occur.

Direct cellular acclimation within the thyroid might still be related to pituitary regulation. For example, TSH has been shown to affect the thyroid cell membrane (Scott, Jay, and Freinkel, 1966) altering its nature and the electrical resistance. Such changes have been shown to initiate pinocytosis (Brandt and Freeman, 1967). Thus, structural modification of the membrane during acclimation (Johnston and Roots, 1964; Scott et al., 1966) might alter the cell's sensitivity to TSH. Simultaneous changes in the lysosomal membranes could alter the relationship between enzyme and substrate or enzyme and cofactor (Dixon

and Webb, 1964). For example, changes in the concentration of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) have been shown to occur with TSH stimulation (Field et al., 1966). Hochachka and Hayes' (1962) work suggests that temperature may have a similar effect.

Additional mechanisms of temperature acclimation occurring at the cellular level may be independent of pituitary function. These could include quantitative changes in enzyme concentration, or qualitative changes in the enzyme. The occurrence of quantitative changes in enzyme activity during acclimation to temperature are common (Prosser, 1962). For example, the levels of a cellular cathepsin of _Rana sp_. and a digestive proteinase of _Helix pomatia_ have been shown to increase during cold acclimation (Precht, 1958).

The evidence of other workers has been reviewed and indicates that quantitative and qualitative changes in enzymes are ways in which the thyroid could acclimate to temperature change. It is possible that other mechanisms such as changes in TSH production by the pituitary or the structural modification of the thyroid cell membrane are involved. A possible relationship, consistent with present knowledge, is that the pituitary mechanism serves to maintain function during the period of acclimation, while direct cellular acclimation to temperature occurs as a more gradual structural and functional modification of the thyroid cells. It is suggested that the role of the thyroid in temperature acclimation of fish may depend in part upon the ability of the thyroid to acclimate to temperature change.

An attempt was made, therefore, to identify and study the thyroid proteinase

of _Lota lota_ with the following objectives:

1. To describe the response of the thyroid proteinase to alterations in temperature _in vitro_ and _in vivo_.

2. To investigate the possibility that the enzyme may be used as an index of thyroid activity.

3. To attempt an evaluation of the role of the thyroid in temperature acclimation.

MATERIALS AND METHODS

The species investigated was the burbot, Lota lota Linnaeus. While subspecific forms have been described frequently, recent work by Lindsey (1956) and Lawler (1963) has shown that they are not valid. The studies were carried out at the University of Alberta, Edmonton, from September, 1963 to May, 1967; most of the work was done between May, 1966 and May, 1967.

A. SOURCE OF FISH

The fish were taken by trap net from Lac Ste. Anne, a large Aspen Parkland lake approximately 80 km west of Edmonton. Reference to map sheet 83G/9 W, Onaway, National Topographical Series 1:50,000 Can., 1st ed., indicates longitude of approximately 114°15' W and a latitude of approximately 53°45' N. Lac Ste. Anne is an extremely shallow (maximum depth about 12 m), highly productive lake which experiences a marked seasonal temperature change. Midsummer temperatures may reach 20 to 25 C whereas winter temperatures average 0 to 2 C. Data collected during the study period indicated that thermal stratification was rare and slight; the temperature between the surface and the bottom differed by only 2 to 3 C. Periodic investigations during the summer of 1966 indicated no significant stratification in oxygen concentration (Langer, 1967). Fig. 15 shows seasonal changes in water temperature at a depth of 3 m.

B. TRANSPORT OF FISH

Live fish were periodically removed from the large trap net in which they had been caught and immediately taken to the Lac Ste. Anne fish research laboratory

in a live tank containing lake water at environmental temperature. At the lakeshore laboratory the seasonal samples described below were taken as quickly as possible. During the sampling period, fish were held in 180 liter barrels of aereated lake water. Fish were secondarily transported to the University of Alberta at Edmonton in 180 liter barrels of oxygenated lake water. During warm weather (summer and early fall) dechlorinated ice was added to the water during transport to the University. When ice had been used it was necessary to hold fish at reduced temperatures (5 to 10 C) for several days after transport.

C. HOLDING CONDITIONS

At the University fish were placed in dechlorinated water in large (720 liter) tanks. Refrigeration equipment was available when needed and air was routinely supplied to all tanks. Some tanks were equipped with a recirculating filtered water system and were used whenever possible but tanks without filters often were used. In the latter case an increased flow of dechlorinated tap water helped keep the tank clean. Small numbers of fish were held occasionally in 150 liter tanks equipped with refrigeration and air lines.

Fish were used as soon as convenient and were not fed. During 1966 fish were held at approximately 5C and 15C from May to August. Casual observation indicated these fish were in as good physical health, though thinner, than fish taken from the lake at the end of the summer. Occasionally problems were encountered with fungus and bacterial infection, Saprolegnia and Furunculosis respectively (Davis, 1956). This infection no doubt resulted from damage to the skin during handling. Infected fish were used rarely. Trial injections of terramycin

were successful in combatting the infection.

D. INJECTION PROCEDURE

For injection, fish were anesthetized when necessary with tricaine methanesulphonate (MS222 - Sandoz Pharmaceuticals, Montreal). Complete anesthesia required several minutes, during which time the fish showed considerable agitation and exertion. When help was available, injections were made into non-anesthetized fish; this was rapid (30 to 60 sec) and appeared to have less adverse effect on the fish. Injections were made intraperitoneally (IP) or intravascularly (IV), as required. Intravascular injections were made into the caudal vein or dorsal aorta with almost no post injection hemorrhage. Usually a 1 ml tuberculin syringe and a 20 or 23 gauge needle were used to make injections of up to 1 ml IP or 0.5 ml IV. Material to be injected (TSH or Pituitary extract) was dissolved in 0.7% NaCl solution which was used also for control animals. Radioisotopes were diluted with distilled water.

E. SAMPLING PROCEDURES

Fish were stunned with a blow on the head. Blood samples were taken immediately from the caudal circulation and the blood was allowed to clot under refrigeration. Twenty four hours later serum was decanted and stored at -15 C. Secondly, 200 mg skin samples were taken, placed in tared 16 x 125mm incineration tubes, capped and later weighed. The thyroid region of the fish was then removed and the ventral aorta and conus arteriosis dissected out and weighed on a torsion balance (Fisher Scientific Co.) to the nearest mg. The samples were then quick-frozen in a dry ice/acetone mixture and stored in tightly capped vials on

dry ice. Upon returning to the university the samples were placed in a deep freeze until analyzed. Finally, data on weight, sex, and degree of gonadal development were taken. The stage of gonadal development was expressed as gonadal somatic index (GSI).

$$\text{GSI} = \frac{\text{gonad weight}}{\text{body weight}} \times 100\%$$

A small consistant error in body weight values was introduced since no correction was made for tissues taken for enzyme analysis or removed during the sampling process.

In radioisotope experiments the same procedure was followed, taking care to avoid cross contamination between tissues. Samples of tissues were placed in tared containers or, alternatively, weighed to the nearest mg and placed in containers.

F. ANALYTICAL TECHNIQUES

1. Enzyme Methods

a) Principle. The method of proteinase assay is essentially that of Anson (1937). After incubation the enzyme substrate mixture is precipitated with trichloroacetic acid (TCA). The TCA soluble products of proteolytic digestion are then isolated. A portion of the product solution is reacted with the Folin Ciocalteu Phenol reagent in an alkaline medium to give a blue color which is proportional to the tyrosine and tryptophane concentration in the solution.

b) Enzyme preparation. The tissue to be extracted was sliced with a razor blade while frozen. A l ml volume of 0.9% NaCl solution was added to each 100 mg of tissue. It was then homogenized with a Teflon pestle glass homogenizer (A. H. Thomas Co.) suspended in crushed ice. After standing for a minimum of one hour the sample was centrifuged at 800 x g for 10 min, after which the supernatant was removed and stored under refrigeration. Improvements in this method were made and most of the enzyme extracts were prepared by the modified method involving the use of a mixture of 1 part of a 1% solution of Triton X-100 (Rohm and Haas) and 9 parts of a 0.9% NaCl solution as the extracting medium. Some extracts prepared very early in the study were centrifuged at 3000 to 7000 x g, forces which could have removed free lysosomes from the supernatant, reducing the enzyme concentration. However, since the tissue had been frozen and then homogenized in saline (both of which are likely to promote the rupture of lysosomes) it is probable that the lysosomes had ruptured, releasing their enzyme into solution. Thus, these centrifugation forces, likely, would not be detrimental since they would have no effect on the enzymes once they were in solution. Results of preliminary studies on methods of extraction are given in appendix 1.

To identify enzymes collected at different seasons and to provide a check against possible storage effects, enzymes were labelled with a two part number. For example, the enzyme called (18/5/66 - 22/4/67) was collected 18 May, 1966 and analyzed 22 April, 1967.

c) Substrate preparation. A 10% hemoglobin solution was prepared as follows: triply washed bovine red cells obtained from the Edmonton plants of Canada

Packers Ltd. and Gainers Ltd. were hemolyzed and dialyzed against distilled water overnight. Hemoglobin content was estimated by one of the methods described below and diluted to 10%. Dilution of the stock solution to 1% with acetate buffer inevitably produced denaturation and precipitation of most of the hemoglobin. However, since the resulting suspension gave consistent results it was used as substrate. Near the end of the study, it was learned that a two stage dilution permitted the preparation of a true hemoglobin solution. Initial buffering of the stock solution to pH 4.0, followed by centrifugation at 3000 x g for 10 min produced a clear solution with a minimal hemoglobin precipitate. Subsequent dilution and adjustment to more acid pH values was then possible. Analysis of enzymes on this substrate was found to give somewhat higher activities.

Care was taken to ensure that the method of substrate preparation did not interfere with comparisons between different samples. All TSH studies utilized substrate prepared by the first method while seasonal studies were analyzed using the newer type of substrate. The new method of substrate preparation presented another problem; solutions greater than 1% at pH 4.0 gelled after a few hours. More concentrated solutions gelled more rapidly and under more acidic conditions, thus limiting the concentrations that could be used effectively. Details of the effects of different substrates on enzyme activity are presented in appendix 2.

Thyroglobulin, used as substrate in some experiments, was prepared by subjecting a crude saline extract of beef thyroids to $(NH_4)_2 SO_4$ fractionation (McQuillan, 1954). The fraction precipitating between 37% and 41% $(NH_4)_2 SO_4$ was dialyzed free of $(NH_4)_2 SO_4$ and frozen or freeze-dried for storage.

A 1% solution in acetate:HCl buffer was prepared for use as substrate. In order to reduce the activity of the residual proteinase the preparation was heated at 55 C for 60 min. This substrate, also, was largely in the form of a suspension.

d) Substrate assay. Initially, hemoglobin solutions were analyzed by the cyanmethemoglobin method (Bausch and Lomb "Spectronic 20" Colorimeter Clinical Methods and Calibrations Manual). Twenty µliter of hemoglobin were mixed with 5 ml of Drabkin's solution. After 10 min the % transmittance of the sample was read in the colorimeter at 540 nm, using Drabkin's reagent alone as a blank. The hemoglobin concentration was read from the calibration chart provided with the colorimeter. Because this method lacked sensitivity, a phenol reagent method was adopted (Anson, 1938). One hundred µliter of hemoglobin, 1 ml of 0.3 N HCl, 2 ml of 0.55 N NaOH, and 10 ml distilled water were mixed together and reacted with 0.6 ml of standard phenol reagent. (Folin Ciocalteu Phenol Reagent, British Drug Houses, diluted according to the manufacturers instructions) Standard solutions consisting of 1 ml portions of solutions containing 0, 250, 500, 750, and 1000 ug equivalents/liter of tyrosine in 0.3 N HCl were mixed with 2 ml of 0.55 N NaOH and 10 ml water and reacted with 0.6 ml of phenol reagent. Twelve minutes after adding the phenol reagent the samples were read against appropriate blanks at 750 nm in the spectrophotometer. Values were converted from tyrosine equivalents to 1% hemoglobin using Anson's 1938 relationship that 0.1 mg of tyrosine produced a color equivalent to 1 mg of hemoglobin.

The two methods used for hemoglobin assay did not give comparable

results, nor did they agree with the gravimetric method of Anson (1938). Appendix 2 shows the relationships. Where critical substrate concentrations were important the phenol reagent method was used.

Thyroglobulin was assayed by the biuret method (Layne 1957) using a standard plasma preparation (Labtrol Dade Reagents Inc.) as a reference standard.

e) Temperature. Analyses were carried out at 0, 5, 10, 13, 15, 20, 25, and 30 C. Routine analyses were done at 30C during the early part of the work and at 25 C in 1967. Temperature control was usually excellent, with a maximum variation of 0.2 C. Temperature controlled water baths were used for most studies. Temperatures below ambient were maintained through the use of a Lauda Ultra Kryomat TK 30 or a refrigerated Warburg water bath (Gilson Medical Electronics). Substrates were preincubated at the assay temperature for 30 min. The duration of incubation of enzyme and substrate was adjusted so that maximum product values could be achieved while still remaining within the period of time during which the hydrolysis was linear.

f) Buffers. An acetate:HC1 buffer was used for most of the assays. Other buffers used were sodium acetate:acetic acid, Na_2HPO_4:KH_2PO_4 (Sørenson), and Tris:HC1 (Gomori). All formulae for buffers were taken from Hale (1958). A Beckman Zeromatic pH meter was used to check buffers and substrates.

g) Procedure for enzyme assay. The basic analytical procedure used two tubes; the first served as the control or reagent blank while the second contained the sample to be analyzed. Each contained 1 ml of buffered hemoglobin.

To initiate the analysis 100 μliter of enzyme were added to each tube. When thyroglobulin was used as substrate the amount of enzyme added per ml of substrate was increased to 250 μliter. The contents of the control tube were precipitated immediately with 2 ml of 0.35 N TCA; this served as a color blank. At the end of the incubation period 2 ml of 0.35 N TCA were added to the sample tube to precipitate the mixture and terminate the reaction. Corrections were made for endogenous enzyme in the substrate preparation by estimating its activity with a second set of tubes which contained no enzyme. A later modification improved the system.

In this modification, enzyme was added to only the assay tube at time zero while the substrate in the control tube was allowed to react with any endogenous enzyme for the duration of the incubation period. At the end of the incubation period both tubes were precipitated within 30 second and then enzyme was added to the control tube. The control tube then served to correct for endogenous substrate activity and residual color material in either the substrate or the enzyme preparation. After addition of TCA the samples were mixed and allowed to stand for at least one hour to ensure precipitation. The contents of each tube were then filtered through a fibreglass filter disc (Reeve Angel glass) using a vacuum filter device (Fisher Scientific Co. Filtrator). Alternatively, centrifugation at approximately 3400 x g was used. In almost all cases each analysis was carried out in duplicate and the mean values of the estimations reported.

h) Colorimetry. One ml of the clear supernatant containing the digestion products was pipetted into a test tube. Two ml of 0.55 N NaOH were added and the contents mixed. A 0.6 ml volume of Folin Ciocalteu Phenol Reagent (British Drug Houses), diluted according to the manufacturer's instructions, was added to control and sample tubes in close succession and the tubes remixed. Twelve minutes later the experimental tube was read in a Hitachi Perkin-Elmer Spectrophotometer (model 139 UV/VIS) at 750 nm, after setting the slit mechanism to 100% transmittance with the control tubes (blanks).

A standard curve was used to convert % transmittance to tyrosine equivalent units. One ml volumes of standard solutions with tyrosine concentrations ranging from 0.01 meq/liter to 0.25 meq/liter in 0.3 N HCl were substituted for samples. Activities were then expressed as tyrosine units/unit time. One tyrosine unit (TU) is defined as the amount of enzyme which produced a color equivalent to that produced by 1 ml of a solution containing 0.01 meq/liter tyrosine, using the Folin Ciocalteu Phenol Reagent as described above.

The sensitivity of the color development to pH and the instability of color with time necessitated careful standardization of technique. Solutions of HCl, TCA, and NaOH were checked by titration against known solutions of NaOH and HCl and adjusted to the required concentration.

i) Isolation of lysosomes. Fresh thyroid tissue was removed from fish and stored on ice until homogenized in 1 ml of 0.25 M sucrose/100 mg tissue. After the residue was removed by centrifugation at 800 x g for 10 min,

the lysosome fraction was collected by centrifugation at 15,000 x g for 20 min (Deiss et al., 1966). The supernatant containing the soluble proteinase was removed and a volume of 0.1% Triton X-100 in 0.8% NaCl, equal to the initial extraction volume, was added to the lysosome fraction. The residue was re-extracted with 0.1% Triton X-100 in 0.8% NaCl. The enzyme content of each fraction was assayed by the techniques described above with the following modification: once TCA was added to the incubation mixture, the tubes were kept in an ice bath to reduce the background color produced when TCA hydrolyzed the sucrose (Wattiaux and DeDuve, 1956).

2. Radioisotope Experiments

$Na^{131}I$ or $Na^{125}I$, diluted in distilled water was administered IV or IP as required. Doses of 20 to 30 μc/fish in a volume of 0.5 ml were used. Standards, consisting of a known fraction of a dose diluted to 50 ml were prepared in duplicate. A measured volume of this was counted with the samples. The counts were corrected for background radiation, sample dilution, and isotope decay. Counting was carried out with a variety of Well Scintillation detectors, all of which had a 2 inch NaI (Tl) crystal with a 33 mm x 17 mm well. In the case of the temperature acclimation experiment the unexpected loss of the automatic sample changer necessitated manual counting. Time limitations prevented the counting of samples for as long as desirable but there was no obvious increase in variability of results when samples were counted either for a minimum of 5000 counts or for 10 minutes.

Tissues of known weight were dissolved in 10% NaOH. Measured portions were counted and counts were corrected for weight of sample and fraction counted. Alternatively, smaller pieces of tissue were hydrolyzed directly in counting tubes and counted, taking care to keep volumes constant. Radioactivity, corrected for background, was expressed as counts/min/g fresh tissue. The ratio of tissue activity to serum activity was calculated as:

$$\frac{\text{tissue count/min/g}}{\text{serum/count/min/g}} = \frac{T}{S}$$

3. Stable Iodide (^{127}I) Analysis

These were carried out by the method of Acland (1957), modified for use in this laboratory by Dr. C.P. Hickman, Jr. In principle, iodide catalyzes the reduction of Ce^{+4} to colorless Ce^{+3} by arsenious acid (Acland, 1957). The addition of brucine sulphate after a measured time terminates the reaction and stablizes the residual color (Grossman and Grossman, 1955). The following procedure was used: One ml of 4 N Na_2CO_3 was combined with 1 ml of serum in an ignition tube. This was mixed carefully, dried at a temperature of 80 to 90 C and incinerated at a temperature of 550 - 570 C for 4 hr with the occasional admission of air to the furnace to ensure complete combustion. The furnace was then allowed to cool and the samples removed. One ml of 2 N HCl and 6 ml distilled water were added to the residue. The contents of the tubes were mixed at regular intervals for 30 min, then centrifuged at 2500 rpm to pack the residue. Duplicate 3 ml volumes of the clear supernatant were pipetted into 14 x 103 mm test tubes for iodide determination. To each tube was added 2.25 ml of arsenious acid solution. Standards were prepared by pipetting 1 ml KI solution into each

of a series of test tubes. Concentrations of I (As KI) ranged from 0.01 to 0.20 µg I/ml. The composition and pH of these were adjusted to be equivalent to serum samples by adding 3 ml of a calibration solution. The preparation of reagents is described in appendix 3.

Arsenious acid (1.25 ml) was added to each standard. All tubes and reagents were placed in a water bath at 37 C for 30 minutes to allow for temperature equilibration. Then, at exactly 30 second intervals, 0.25 ml of a Ce^{+4} solution was added to each tube and the contents mixed with an air capillary. After 12.5 minutes 1 ml 0.5% w/v brucine in 5% H_2SO_4 was added to stop the reaction and the contents were again mixed with an air capillary. At the conclusion of the series the tubes were heated in a boiling water bath for 30 min to develop the full color of the brucine - Ce^{+3} complex.

Samples were read against a distilled water blank at 540 nm in a spectrophotometer. The standard curve was drawn and % transmittance readings of the unknowns were converted to µg iodide. Calculations for dilution and sample size were made and results expressed as µg of iodide/100 ml of serum.

Weighed skin samples of about 0.2 g were hydrolyzed in 1 ml ethanol and 1 ml of 4 N KOH (McNabb, 1963). Since KOH serves to prevent loss of iodide during incineration (Foss, Hankes, and Van Slyke, 1960) no Na_2CO_3 was added. After drying, these samples were treated in the same way as serum. During the course of this work it was discovered that KOH depressed the colorimetric reaction. To correct for this effect, a curve was constructed which related

the true ^{127}I concentration for a given % transmittance to the apparent value. By finding the point on the y axis equivalent to the incorrect value it was possible to read the corrected value from the x axis. Skin values were expressed as ug iodide/g of fresh tissue.

In all analyses reagent blanks, distilled water controls, and standard serum controls (Iodotrol - Dade Reagents Inc.) were run in duplicate. The use of distilled, demineralized water for the preparation of reagents and rinsing of glassware was found to be essential. Calibrated syringes permitted accurate and rapid addition of reagents. Appendix 3 gives the brands of reagents found suitable for iodide analysis.

Statistical tests used are described in Snedecor 1956 and Siegal 1956.

RESULTS

CHARACTERISTICS OF THYROID PROTEINASE

The relative activity of a proteinase preparation (11/1/66-12/1/66) on hemoglobin and thyroglobulin substrates was studies by incubating enzyme and substrate for periods of 1, 2, and 3 hr at 30 C. Fig. 1A shows that the hydrolysis of hemoglobin (curve A) proceeds in a linear fashion for 2 to 3 hr. In contrast, thyroglobulin hydrolysis (curve B) ceases to be linear after 1 hr. The greater susceptibility of hemoglobin to proteinase digestion is indicated by the ratio of the initial digestion rates.

$$\frac{\text{hemoglobin digestion/hr}}{\text{thyroglobulin digestion/hr}} = \frac{55\ \text{TU/hr}}{25\ \text{TU/hr}} = 2.2$$

The thyroid proteinase appears to be relatively ineffective in its action on beef thyroglobulin substrate.

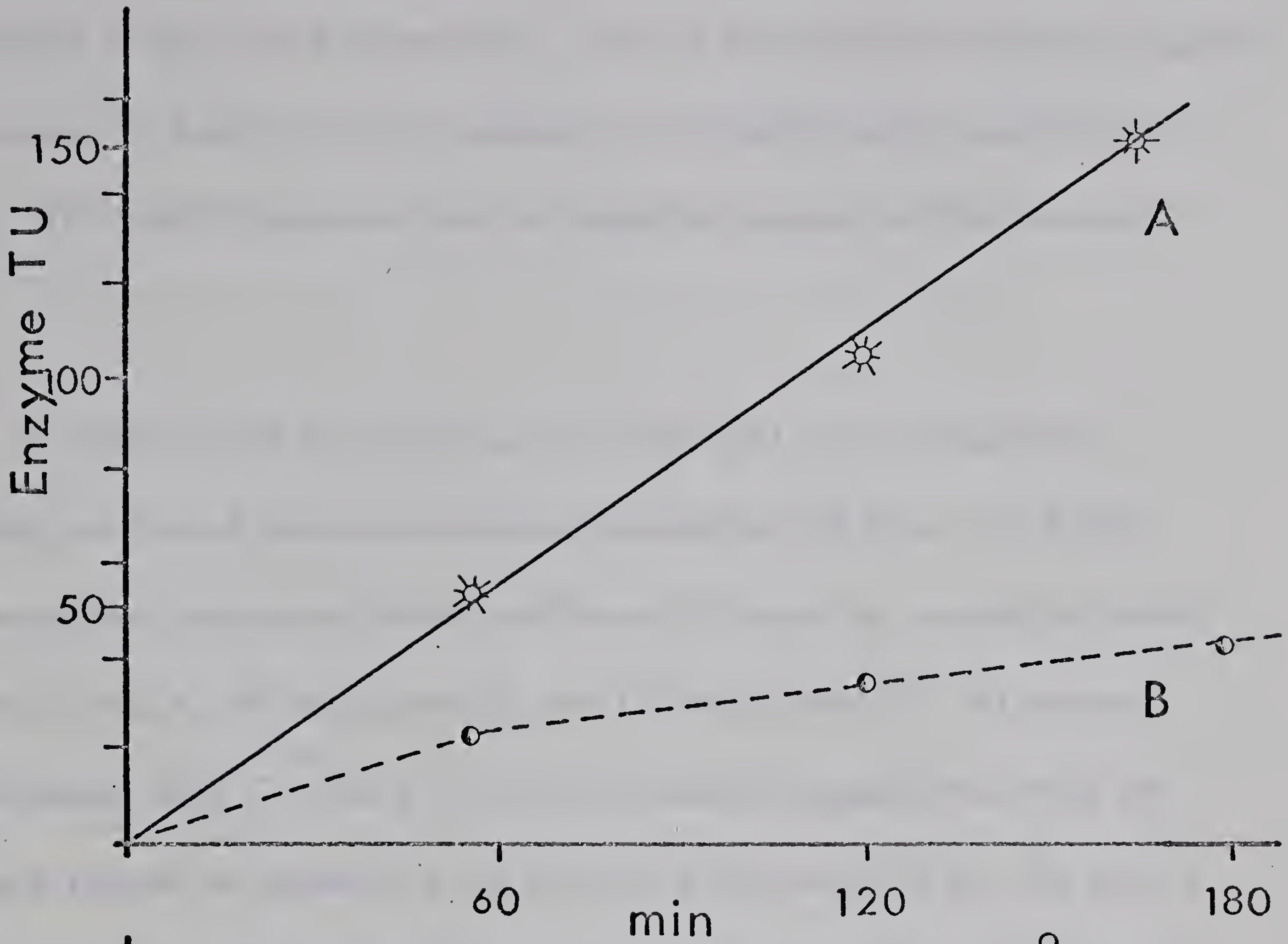
Fig. 1B represents the results obtained during attempts to show that the proteinase activity was directly related to enzyme concentration. Two studies (curves A and B) were carried out on hemoglobin substrate using different enzyme preparations, whereas a single study (curve C) used thyroglobulin substrate. Curve A indicates the expected results; an essentially linear response occurs over a range of enzyme concentration from 10 μliters/ml to 200 μliters/ml of proteinase (18/5/66 - 29/3/67). Curve B (18/5/66 - 18/7/66) shows a gradual decline in activity/unit volume enzyme added as the enzyme concentration is increased from 50 μliters/ml to 500 μliters/ml. The basic difference in the two curves appears to be related to the larger volumes of enzyme added to

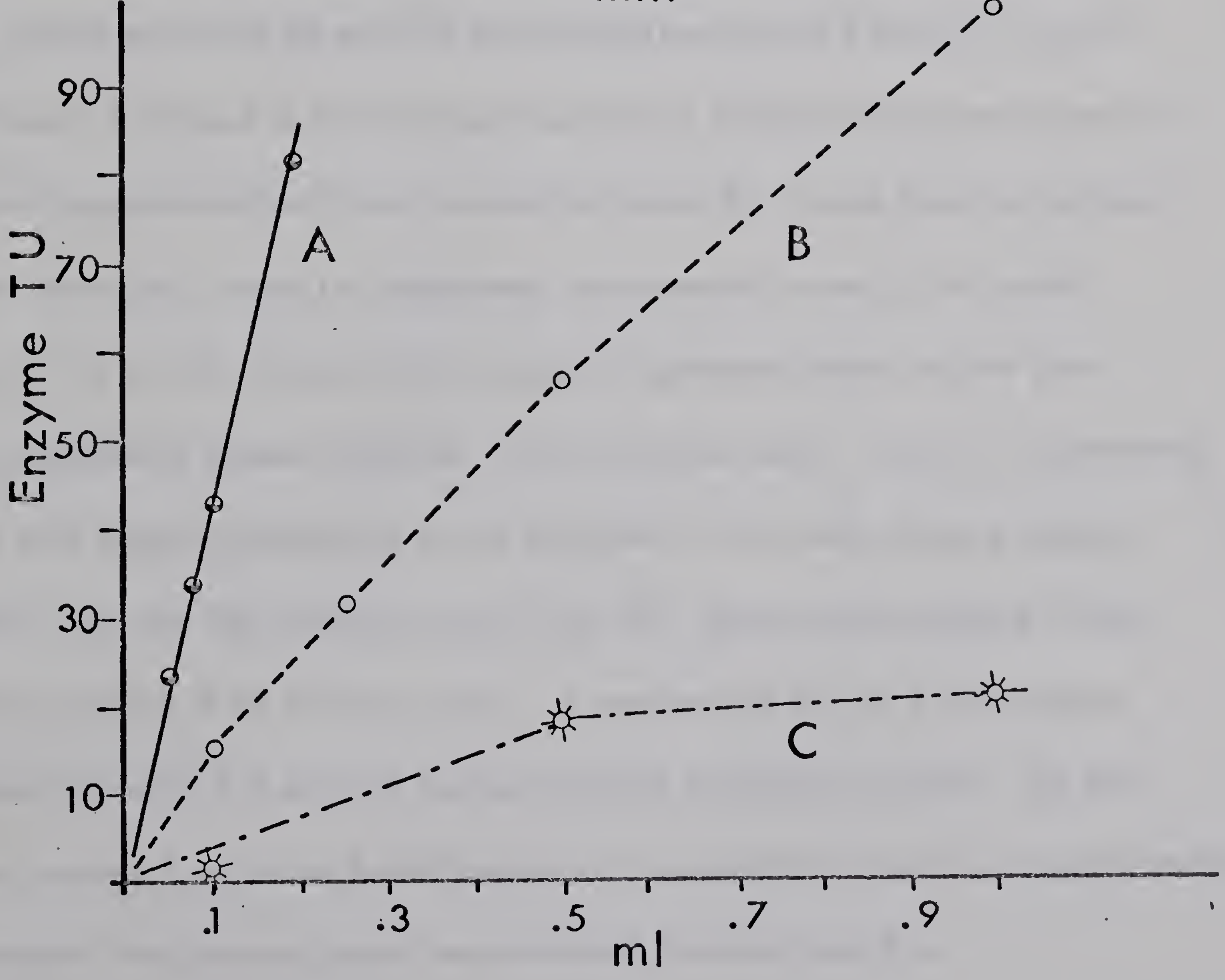
Fig. 1A.

The relative susceptibility of hemoglobin substrate (curve A) and thyroglobulin substrate (curve B) to hydrolysis by burbot thyroid proteinase at pH 2.5 and 30 C.

Fig. 1B.

The effect of enzyme concentration on enzyme activity,
(hemoglobin substrate - curves A and B)
(thyroglobulin substrate - curve C)
Curves A and B based on different enzyme preparations. Volume of enzyme added to obtain different final enzyme concentrations is plotted on the X axis. Incubated at pH 2.5 and 30 C.


Enzyme TU
150
100
50
A
B
60
120
180
min


Enzyme TU
90
70
50
30
10
A
B
C
.1
.3
.5
.7
.9
ml

the substrate in the curve B experiment. Curve C (thyroglobulin substrate) suggests that increases in digestion rate in response to increased enzyme concentration is limited. This radical departure from the expected response will be discussed below.

The effect of pH on enzyme activity (Fig. 2A) was investigated by incubating portions of the same proteinase preparation (18/5/66 - 12/6/66) with hemoglobin substrate buffered to different pH values for incubation periods of 45 min (curve A), 90 min (curve B), and 135 min (curve C). All samples were incubated at 30 C. Curve A (45 min incubation) suggests that there are three peak regions for digestion at pH 2.5, pH 3.25, and pH 4.0. The pH 4.0 peak is visible also after 90 and 135 min incubation (curves B and C). In contrast, the pH 3.25 peak is not distinguishable after 135 min incubation (curve C) and is only suggested after 90 min incubation (curve B). Since these curves were based on individual values the experiment was repeated to verify the results of curve A. Fig. 2B, curve B, shows essential agreement when enzyme from burbot collected in summer (18/5/66 - 10/11/66) was used. Curve A, representing enzyme from burbot collected in winter (8/10/66) - 2/11/66), shows a pattern essentially like the 135 min curve (A) in Fig. 2A. Both studies reported in Fig. 2B were incubated at 30 C for one hour. It appears that the pH 3.25 optimum is masked by the pH 2.5 activity during extended incubation periods. The pH curve of enzyme from winter burbot appears to be essentially like the curves obtained when enzyme from summer burbot was incubated for more than 2 hr.

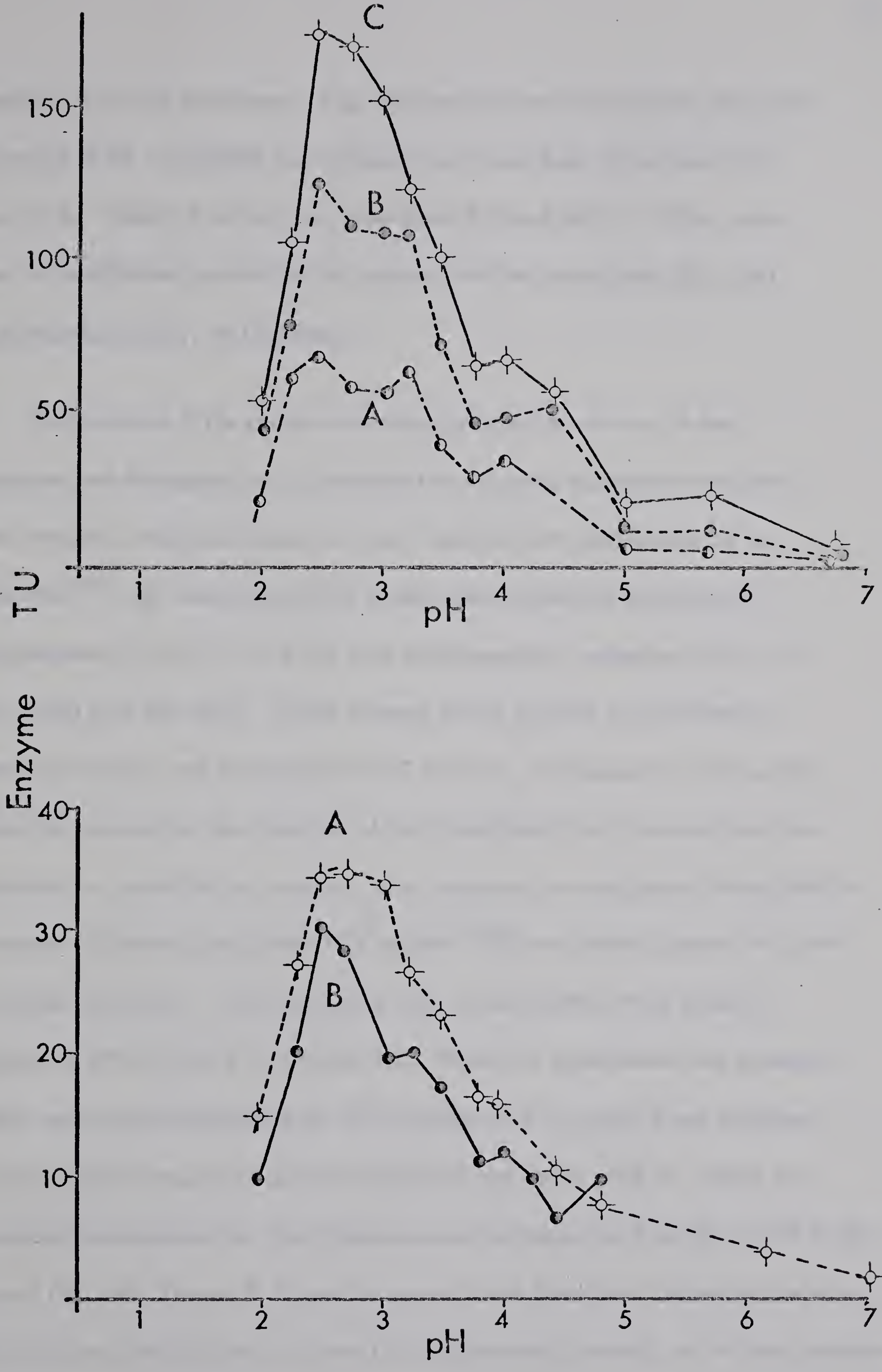
Studies were carried out to determine the optimum pH for the hydrolysis of

Fig. 2A.

Activity of thyroid proteinase extracted from summer fish on hemoglobin substrate at different pH values. The study indicates the effect of incubation period on pH curve. Curve A represents an incubation period of 45 min, curve B 90 min, and curve C 135 min. Temperature 30 C.

Fig. 2B.

Activity of thyroid proteinase at different pH values for enzyme from burbot caught in the winter (curve A) and from burbot caught in the summer (curve B) on hemoglobin substrate. The incubation period in both studies was one hour at 30 C.


Enzyme TU
C
B
A
150
100
50
1
2
3
4
5
6
7
pH
A
B
40
30
20
10
1
2
3
4
5
6
7
pH

thyroglobulin by fish proteinase. Fig. 3A shows the results obtained when fish enzyme (18/5/66 - 20/6/66) was allowed to act upon beef thyroglobulin for 6 hr at 30 C. Peaks of activity are seen at pH 3.0 and pH 4.5. Thus, there appear to be different optima for the enzyme with the hemoglobin (Fig. 2A) and thyroglobulin (Fig. 3A) substrate.

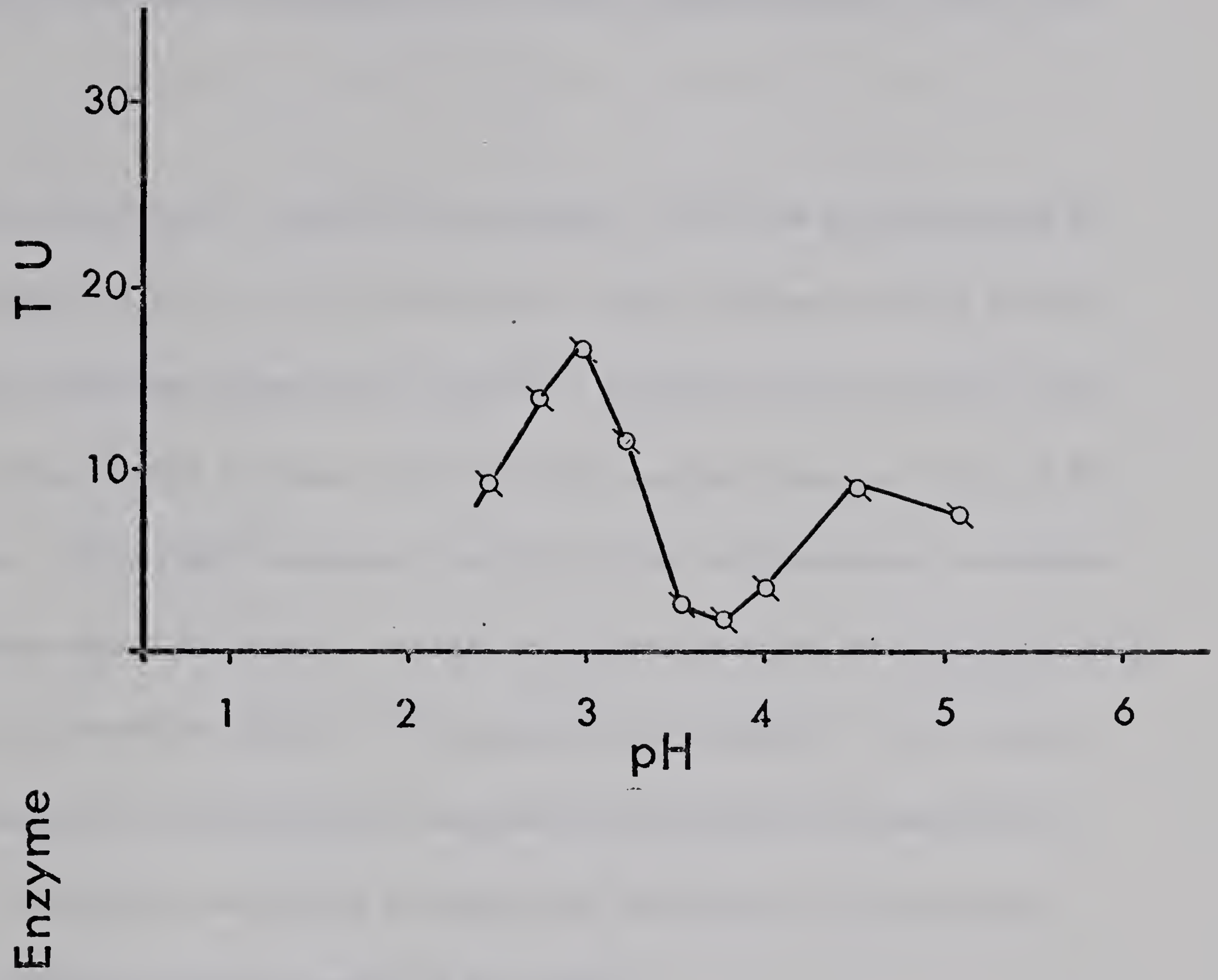
To determine if the results were being affected by the use of beef hemoglobin and thyroglobulin as substrates the following experiment was done. Burbot thyroids, which had been previously labelled with radioiodide by injecting Na ^{125}I IP, were killed after 3 days and radioactive thyroglobulin and proteinase (13/1/67 - 13/1/67) were simultaneously extracted with 0.1% Triton X-100 in 0.8% NaCl. Equal volumes of the extracts were buffered to different pH values and incubated at 20 C for 4 hr. The amount of TCA soluble radioiodide released by the digestion of the thyroglobulin by the proteinase was determined in a scintillation counter, after removing the undigested thyroglobulin. The amount of product (count/min TCA soluble ^{125}I) was plotted against the pH of the incubation mixture. The results (Fig. 3B, curve A) show three peaks of hydrolysis at pH 3.0, pH 3.5, and pH 5.0. When the experiement was repeated with the same extract incubated for 12 hr instead of 4 hr, curve B was obtained. This shows optimal regions of activity at pH 3.0 and above pH 5.0. There are two possible explanations for the differences seen between the 4 hr (A) and 12 hr (B) curves of Fig. 3B. The pH 3.5 peak (curve A) could have been caused by the variability which resulted from the low level of radioactivity counted, or the lack of observations in the region pH 3.0 to pH 3.5 of curve B may have permitted the pH 3.5 peak to

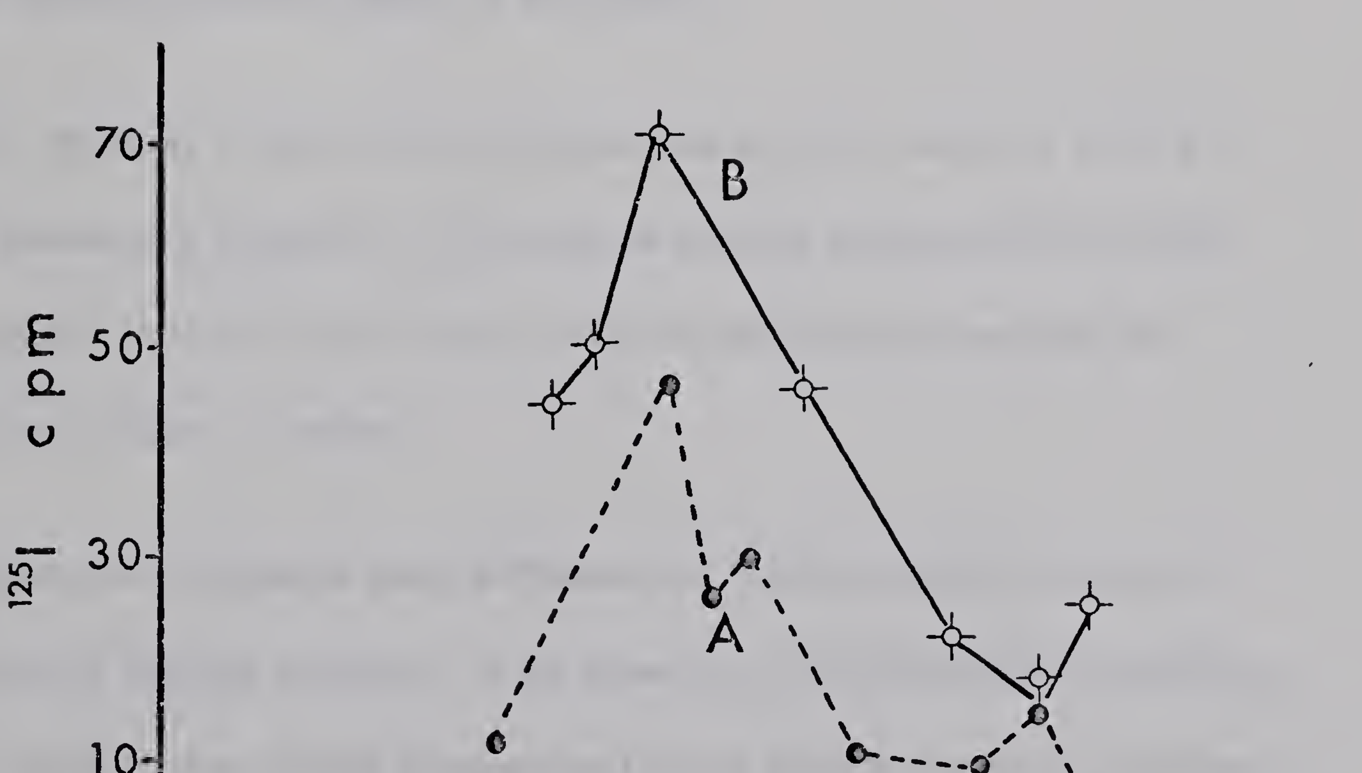
Fig. 3A.

Activity of burbot thyroid proteinase at different pH values using beef thyroglobulin as substrate. Incubations at 30 C for 6 hours.

Fig. 3B.

Rate of autolysis of ^{125}I labelled burbot thyroglobulin by burbot proteinase at different pH values. Activity measured by the amount of TCA soluble radioiodide liberated during incubation period. Lower curve (A) represents 4 hr incubation; upper curve (B) 12 hr. Both studies done at 20 C.


Enzyme T U
30
20
10
1
2
3
4
5
6
pH


^{125}I c p m
70
50
30
10
B
A
1
2
3
4
5
6
pH

pass unnoticed. These data indicate that fish proteinase may show differences in optimal pH values when thyroglobulin from fish and from beef are used as substrates.

One of the basic concepts of enzymology is that low concentrations of substrate may limit the activity of an enzyme. Fig. 4A shows that the activity of a thyroid proteinase preparation (18/5/66 - 3/4/67) increases rapidly as the substrate concentration increases until the latter reaches a level of 0.6 to 0.8% hemoglobin. Only slight increases in activity occur with increases in substrate concentration above this level. In order to achieve near maximum reaction rates substrate concentrations of about 1% hemoglobin are required. If the results of experiments are to be compared, constant concentrations of substrate must be used. Precautions were taken to ensure that differences in substrate concentration did not affect the results of the studies.

Fig. 4B shows relative levels of proteinase activity measured at pH 2.5 in several tissues of a single fish. The enzyme activity extracted from thyroid tissues is greater than that from blood or skin but significantly less than that found in liver, kidney, or spleen.

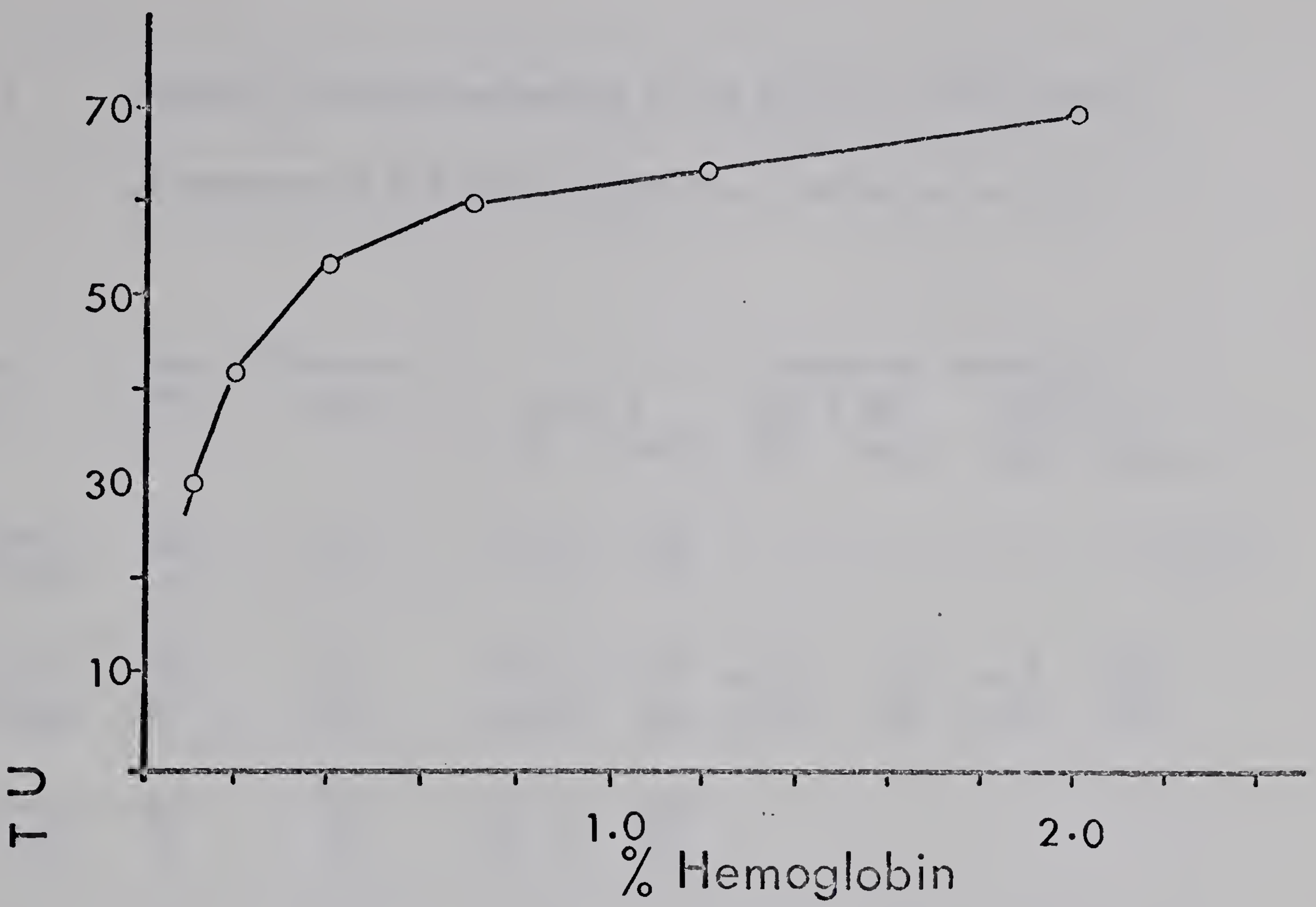
Mammalian cathepsins have differences in thermal stability which are characteristic of specific enzymes. In an attempt to identify the fish proteinase, its thermal stability was studied by exposing thyroid enzyme extracts to selected temperatures for short periods of time. Table 1 presents the data obtained. Brief incubation (5 min) at 50 C completely inactivated the enzyme. Exposure

Fig. 4A.

Effect of hemoglobin substrate on thyroid proteinase activity. Incubated at pH 2.5 and 30 C.

Fig. 4B.

Activity of proteinase in different organs determined with hemoglobin substrate at pH 2.5 and 30 C.


70
50
30
10
T U
1.0
2·0
% Hemoglobin

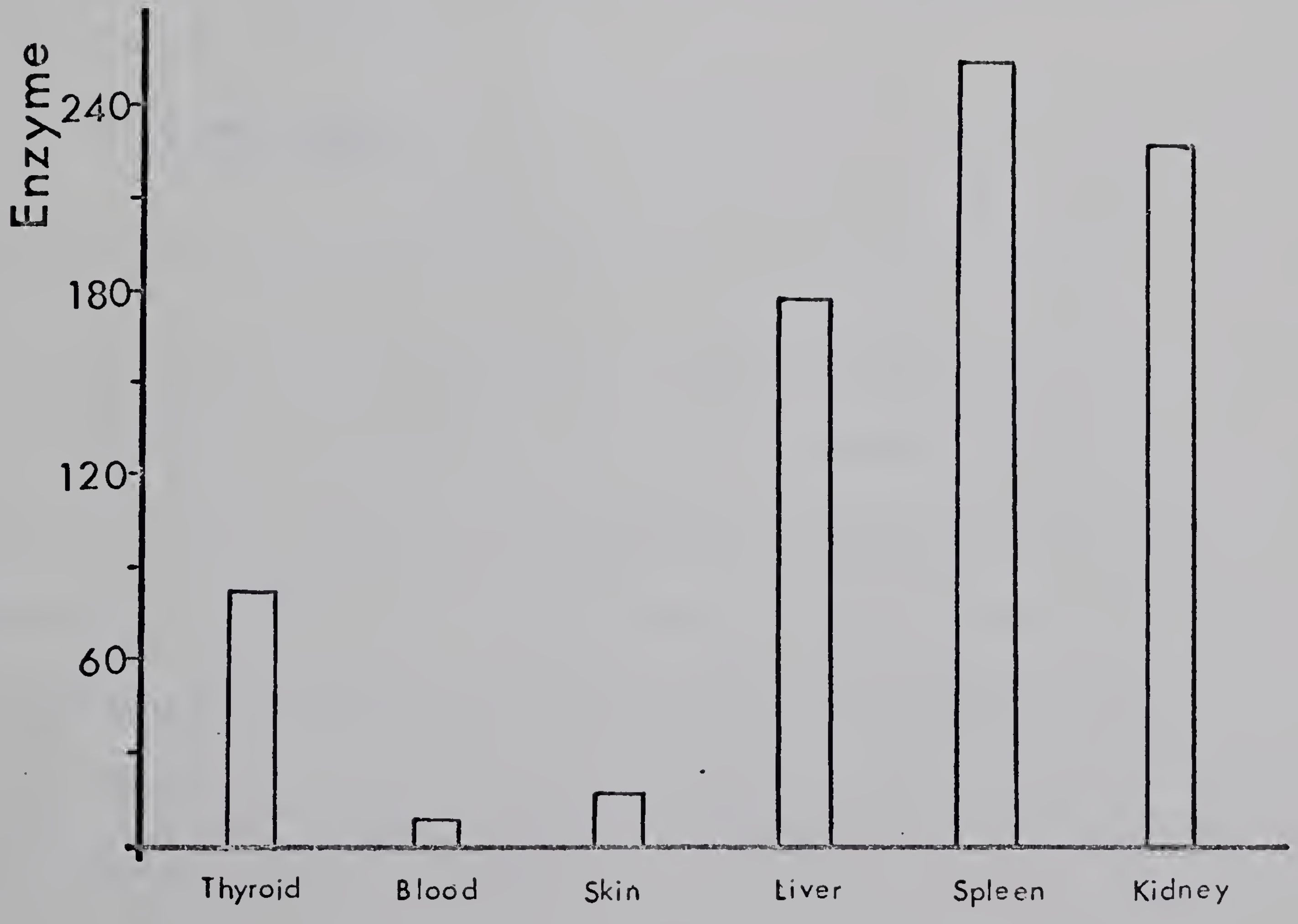

Enzyme
240
180
120
60
Thyroid
Blood
Skin
Liver
Spleen
Kidney

Table 1. Stability of thyroid proteinase of the burbot at combinations of temperature and time; analyses were carried out at 20 C.

Enzyme Source	Temp °C	Duration min	Residual Activity pH 2.5 TU	pH 2.5 %C	pH 3.25 TU	pH 3.25 %C	pH 4.5 TU	pH 4.5 %C	n
(18/5/66)	20	5	37.5	100	-	-	-	-	2
(19/10/66)	50	5	0	0	-	-	-	-	2
(18/5/66)	20	10	48.3	100	40.2	100	14.5	100	1
(26/11/66)	37	10	40.2	83	33.5	83	11.0	79	1
(18/5/66)	20	10	32.2	100	-	-	-	-	3
(17/11/66)	37	10	26.2	81	-	-	-	-	3
(1/3/67)	20	60	37.5	100	26.3	100	24.0	100	2
(1/3/67)	30	60	27.5	73	17.8	68	16.5	69	2

C Control Value

to 37 C for 10 min produced an average 18% inactivation. Heating at 30 C for 60 min produced an inactivation of about 25%. Fish proteinase is much more temperature sensitive than mammalian enzymes.

Further attempts were made to identify the enzyme by determining if its activity was affected by dialysis and by examining the response of the proteinase to a variety of cofactor materials. Table 2 shows that dialysis against running tap water increased the activity of the proteinase (18/5/66 - 20/10/66); dialysis against a 0.9% NaCl solution or distilled water either had no effect or was slightly inhibitory. This study suggests that the addition of a low molecular weight factor may have occurred. After dialysis against ethylenediamine tetra acetate (EDTA) little activity remained, indicating that a metal ion may be essential for proteinase function.

The results obtained when these factors were examined in more detail, using proteinase preparation (9/4/67 - 9/4/67), are presented in Table 3. Portions of the extract were analyzed under standard conditions (control A) and with substrate solutions which contained various additives at a final concentration of 0.01 M. A portion of the untreated control extract was stored in the refrigerator to be analyzed at the same time as the dialysis sample. This was called control B. Upon being assayed under the same conditions as had been used for control A, a loss of activity was observed. This was apparent only when the assay was done at pH 2.5.

Table 2. Activity of the thyroid proteinase at pH 2.5, hemoglobin substrate, after various dialysis treatments.

Enzyme	Treatment	Control TU/hr	Experimental TU/hr
(18/5/66) - (20/10/66)	Tap water dialysis	52.5	72.0
(18/5/66) - (17/11/66)	Dialysis vs. 0.7% NaCl	31.5	31.0
	Dialysis vs. dist. water	31.5	28.0
	Dialysis vs. 0.01M EDTA	31.5	2.0

Table 3. Activity of thyroid proteinase at different pH values, showing the effect of various treatments.

3A Effects of storage and dialysis

Treatment	pH 2.5		pH 3.25		pH 4.0	
	TU	%	TU	%	TU	%
Control A	96.8	100.0	67.5	100.0	56.3	100.0
Control B	90.5	93.5	69.5	103.0	56.3	100.0
Control B	90.5	100.0	69.5	100.0	49.5	100.0
Dialyzed dist. water	88.0	97.2	65.2	93.8	53.8	87.9

3B Effects of metals and EDTA

Treatment	pH 2.5		pH 3.25		pH 4.0	
	TU	%	TU	%	TU	%
Control A	96.8	100.0	67.5	100.0	56.3	100.0
EDTA	89.5	92.5	66.3	98.2	53.8	95.6
Ferrous Ammonium Sulphate	67.0	69.2	48.0	71.0	40.3	71.6
MMI	11.0	11.4	8.5	12.6	11.3	20.1

3C Effects of sulfhydryl reagents

Treatment	pH 2.5		pH 3.25		pH 4.0	
	TU	%	TU	%	TU	%
Control A	96.8	100.0	67.5	100.0	56.3	100.0
Iodoacetate	48.5	50.1	44.3	65.6	33.3	59.1
Glutathione	20.8	21.5	21.8	32.3	24.5	43.5
Cysteine	249.0	257.2	192.0	284.4	157.5	279.8

Distilled water dialysis (Table 3A) reduced the activity to a small extent as compared to control B. Table 3B shows that when (EDTA) was added to the incubation media a slight loss of activity occurred. The addition of ferrous ions (Table 3B), supposedly a specific activator for a hemoglobin splitting proteinase (Snoke and Neurath, 1950), was found to inhibit the enzyme. Since chromatographic analyses of autolyzed thyroglobulin was planned, which would require the use of 1-methyl-2-mercapto imidazole (MMI) to prevent deiodination, the effect of this substance on the proteinase was tested, and found to be inhibitory (Table 3B). The effects of the sulfhydryl reagents tested are presented in Table 3C. Cysteine produced a significant and uniform stimulation while iodacetate and glutathione were strongly inhibitory. Glutathione inactivation was greatest at pH 2.5 and least at pH 3.5.

In summary, the data presented in this section have shown that fish thyroid proteinase has a much greater activity on hemoglobin than on thyroglobulin and that beef and fish thyroglobulin may not be acted upon in the same way. Further differences between fish and mammalian enzymes are indicated by the lower inactivation temperature found for fish enzyme. With hemoglobin substrate, the increase in enzyme activity as enzyme concentration is increased, shows a linear relationship at low concentrations of enzyme only. Fish proteinase has only limited activity on beef thyroglobulin and shows a significant decline in the activity per unit enzyme as enzyme concentration is increased, suggesting that some inhibitory material may be present in the enzyme preparation. In general, dialysis experiments failed to show the presence of an inhibitor substance when

assayed on hemoglobin substrate. These studies did suggest that metal ions were essential for enzyme function. Results with sulfhydryl reagents indicate the presence of a thiol proteinase.

These observations suggest that more than one enzyme is present. This is indicated by the results of the pH activity curves, which show three regions of activity on hemoglobin substrate. For convenience, each pH peak will be considered to represent theoptimum for a separate enzyme. Differences between enzymes extracted from fish caught in summer and in winter appear in the pH curves also.

Biological Role of Thyroid Proteinase

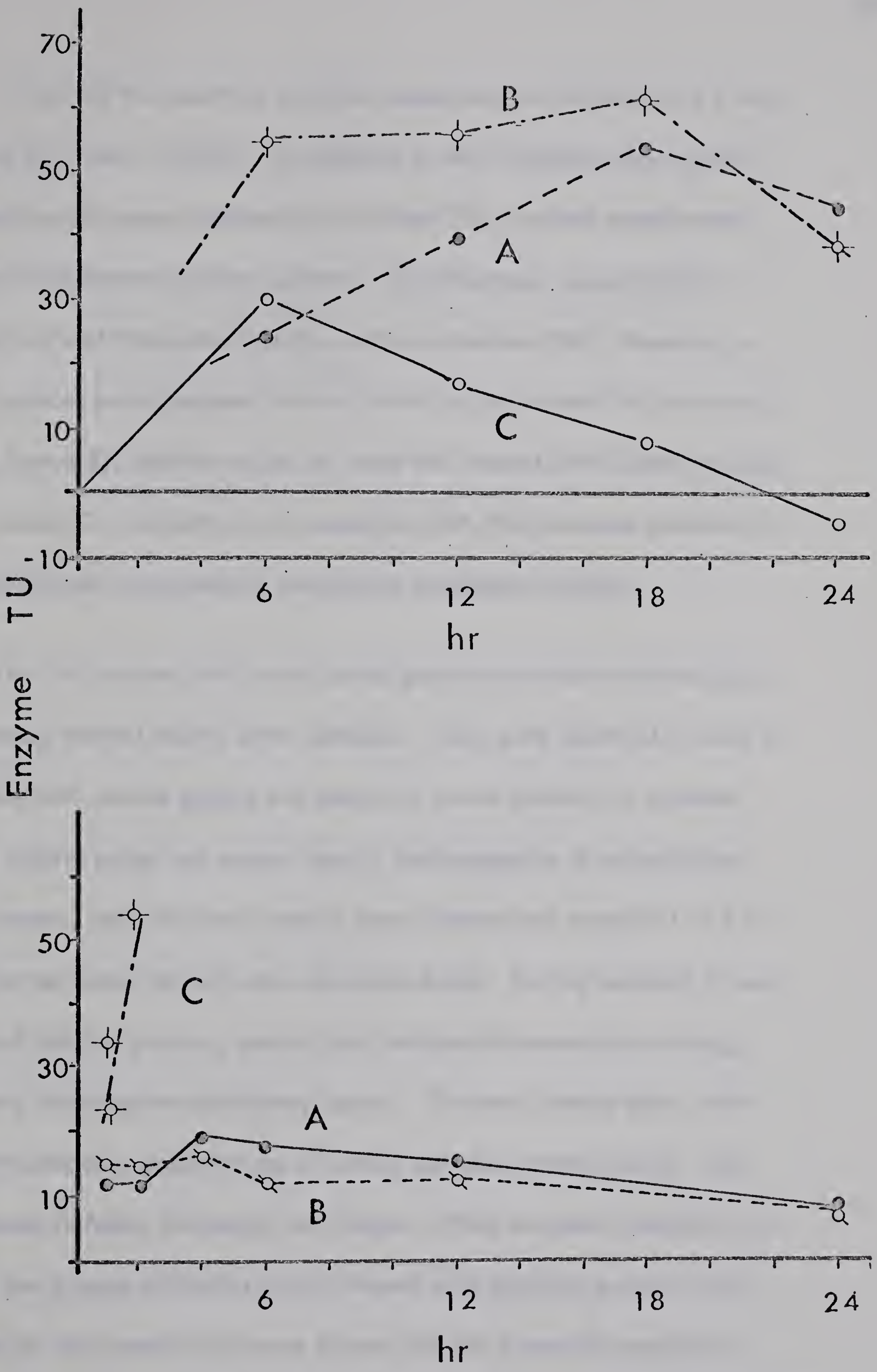
If the proteinase under investigation is thyroidal in origin its activity should respond to TSH stimulation. A series of studies was carried out in which extracts were made of thyroid tissue from fish which had been injected with mammalian TSH. The activity of the extracts was assayed on hemoglobin substrate at pH 2.5. The change in activity of the proteinase at various times after TSH injection into burbot held at 14 to 15 C is shown in Fig. 5A (13/10/66 - 15/10/66). The control values from saline injected burbot (curve A) steadily increased to a maximum at 18 hr while the experimental fish (curve B) showed a rapid increase in enzyme activity and attained a very high value by 6 hr. By 24 hr both groups showed decreased levels of enzyme activity. The net stimulation (activity of enzyme from TSH injected fish minus activity from control fish) is seen in curve C.

Fig. 5A

Effect of TSH injection into burbot upon the proteinase activity of thyroid tissue. Curve A - values for saline injected control fish. Curve B - values for TSH injected experimental fish. Curve C - Curve B minus Curve A represents the increase in activity of experimental burbot enzyme over that of control values. Experimental temperature 14 C. Hemoglobin substrate at pH 2.5 and 30 C.

Fig. 5B

Effect of TSH injection into burbot upon the proteinase activity of the thyroid tissue. Curve A - values for saline injected control fish. Curve B - values for (TSH) injected fish. Curve C - values for fish from the same population injected with a crude extract of burbot pituitary. Conditions as in 5A.


70
50
30
10
-10
B
A
C
6
12
18
24
hr
Enzyme TU
50
30
10
C
A
B
6
12
18
24
hr

In Fig. 5B the results of a similar experiment on fish held at 5 C are presented (8/11/66 - 5/4/67). In addition to NaCl injected controls and the experimental groups injected with purified TSH a second experimental group received burbot pituitary extract. No differences occurred with treatments or with time after injection with mammalian TSH. However, a marked contrast exists between the low levels for the control fish (curve A), TSH fish (curve B), and the values for three fish treated with burbot pituitary extract (curve C). In contrast to mammalian TSH, fish hormone produced a highly significant stimulation of the thyroid proteinase activity.

The fish injected with crude burbot pituitary extract exhibited signs of respiratory distress shortly after injection. They were observed to swim to the surface with mouths gaping and opercular covers spread in a response which is typical under low oxygen levels, and suggestive of asphyxiation. For this reason, only the three animals were injected and sampled 1 to 2 hr after injection since the fish were obviously dying. During sampling it was discovered that the pituitary extract had initiated intravascular clotting, completely blocking the circulatory system. The small sample sizes, with different variances, made the use of normal statistical tests invalid. The non-parametric Mann Whitney U test (Siegal, 1956) was used, therefore, to compare the enzyme activities of fish treated with pituitary extract to the activities of the control fish (curve A) and TSH fish (curve B) sampled at 1 and 2 hr. This showed that the differences were highly significant (P = 0.028).

Table 4. Effects of asphyxiation on the activity of the thyroid proteinase of the burbot.

Group	Sample size	Mean TU	Range TU
Control	4	27.8	14.3-43.8
Asphyxiation	4	21.8	14.3-26.5

A series of TSH studies was done during the summer and fall of 1966 on fish held in the laboratory for about 2 weeks at temperatures ranging from 12 to 16 C. The fish were sampled 6 hr after receiving mammalian TSH. The results of these experiments are presented in Fig. 6. The numbers below the points indicate sample sizes in both the control and experimental groups. In every case the mean thyroidal enzyme activity was greater for the TSH group. Individual values exhibit considerable overlap and the differences in the means are not statistically significant. In addition to the trend for the TSH treated fish to show higher enzyme activities, two other trends are demonstrated. The effect of TSH injection is greatest in the early summer and declines during the fall. At the same time the thyroids of control fish show a gradual increase in proteinase activity. This suggests an increased production of TSH by the pituitary has occurred so that thyroids are being stimulated already and therefore have less capacity to respond to the additional stimulation of exogenous TSH injections.

In April a pituitary extract which had been frozen after preparation was thawed and used in a study similar to those described in the preceeding paragraph. The controls in this study did show a slightly higher mean than the experimentally treated fish but the difference was not significant. These observations are shown also in Fig.6. In the June TSH experiment (Fig. 6) one fish received TSH which had been frozen; it was the only experimental fish in this study that did not show enzyme stimulation, suggesting that the hormone may have been inactivated by freezing. The values for this fish are plotted separately in Fig. 6.

Fig. 6

Effect of TSH injection into burbot upon the thyroid proteinase at different times of the year. All fish sampled 6 hr after injection and analyzed on hemoglobin substrate at pH 2.5 and 30 C.

Fish were held at 12 - 16 C in all cases except in April, when they were held at 4C.

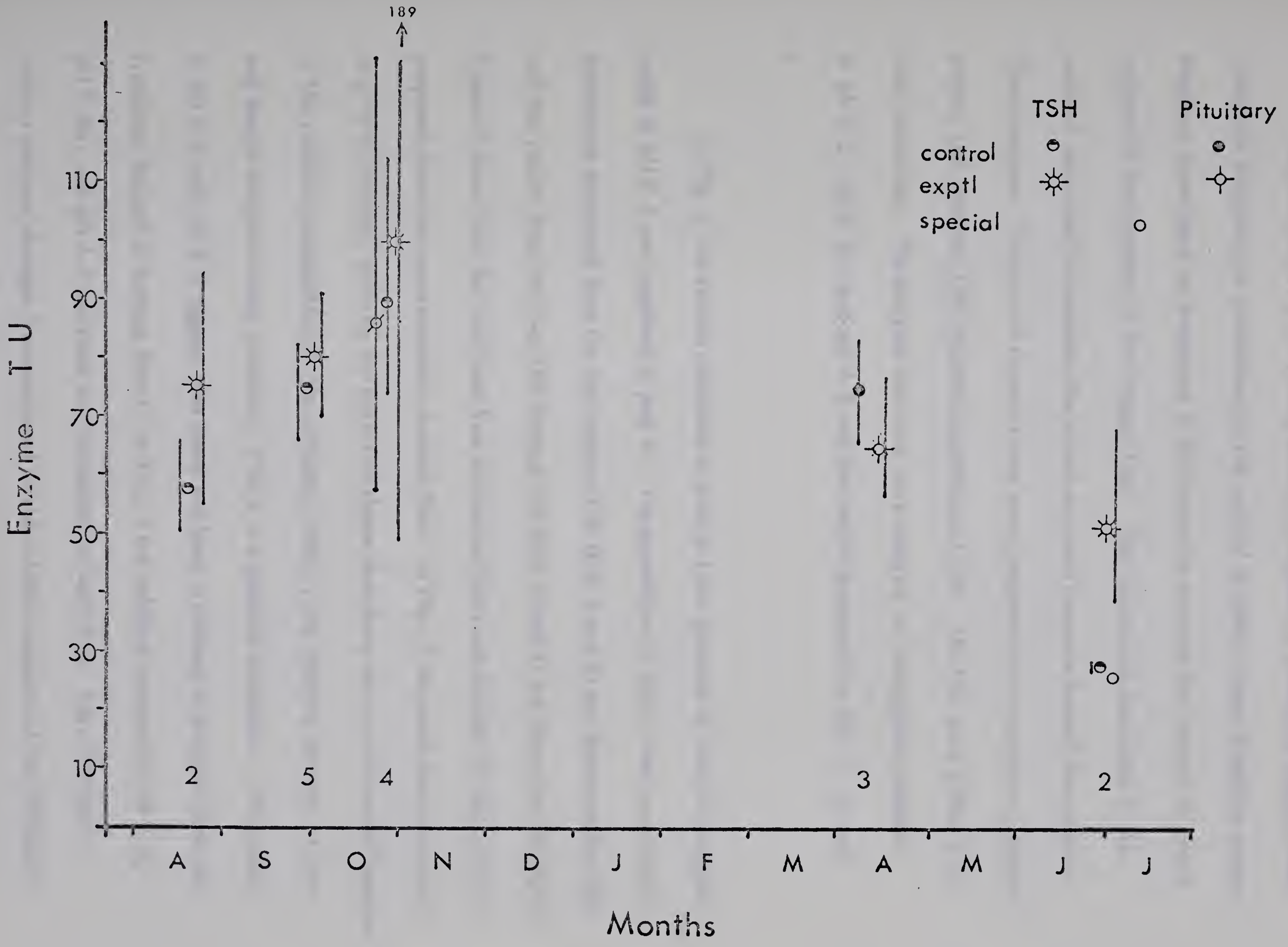
◓ mean values for control fish

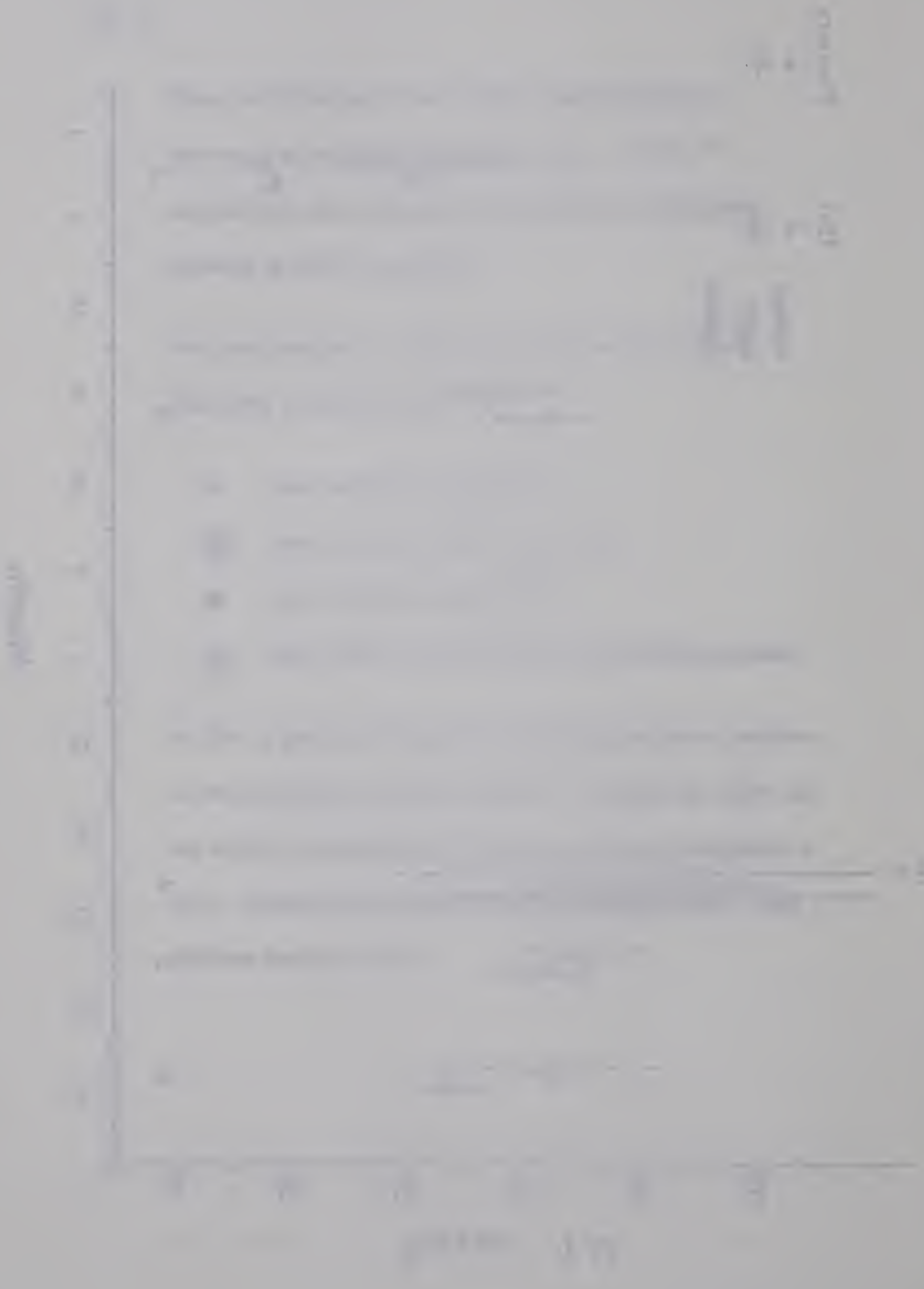
☼ mean values for TSH injected fish

● mean values for control fish

⌖ mean values for fish injected with pituitary extract

In Oct. a group of uninjected controls was studies in addition to the usual saline injected controls. A single fish which was not strictly comparable to the others is plotted individually in July. Numbers below points indicate the sample size. Bars indicate range of values.


189
TSH
Pituitary
control
exptl
special
110
90
70
50
30
10
Enzyme T U
2
5
4
3
2
A
S
O
N
D
J
F
M
A
M
J
J
Months



Wollman et al. (1964) have shown thyroglobulin hydrolysis begins when lysosomal enzymes are transferred to the colloid droplet, where digestion occurs. Attempts were made to determine if TSH acted to increase the amount of active enzyme at the expense of the storage form. The procedures described in the methods were used to separate the stored enzyme (lysosome bound) from active (free) enzyme. Extracts of thyroid tissue were prepared from two saline injected control fish and two TSH injected experimental fish. The fish were killed 7 hr after injection. The enzyme fractions were assayed on hemoglobin substrate at pH 2.5, pH 3.25, and pH 4.0 and the results presented in Fig. 7, 8, and 9.

In Fig. 7 the results obtained at each pH are grouped so that all analyses made at pH 2.5 are reported in part A. The quantities of total, free, and bound proteinase extracted from the two control fish (fish 1 and 2) are shown on the left and the results from the two TSH treated fish (fish 3 and 4) are shown on the right. Figure 7 shows that the total and free enzyme activity are greater in the extracts prepared from the experimentally treated fish. In Fig. 8 the same data expressed as a % of the total activity for each fish shows that there are no obvious differences in the relative composition of the extracts, that is, the relative amounts of free and bound enzyme remain constant. Fish 4 is a possible exception. The results at pH 2.5 and pH 4.0 suggest that there has been a release of enzyme from the lysosomes (bound or storage form). In Fig. 9 the relative amounts of pH 2.5, pH 3.25, and pH 4.0 enzymes are compared for each fish. Fig. 9A shows that no obvious changes have occurred in the relative amounts of the different

Fig. 7A

The activity at pH 2.5 of total proteinase (T), free enzyme (F), and lysosomal bound (B) enzyme for fish 1, 2, 3, and 4 is shown. Fish 1 and 2 were saline injected controls; fish 3 and 4 received TSH 6-7 hr before being killed.

Values are means of duplicate determinations on hemoglobin substrate at 25 C.

Fig. 7B

The results of the same study with analysis being carried out at pH 3.25

Fig. 7C

The results of the same study with analysis being carried out at pH 4.0

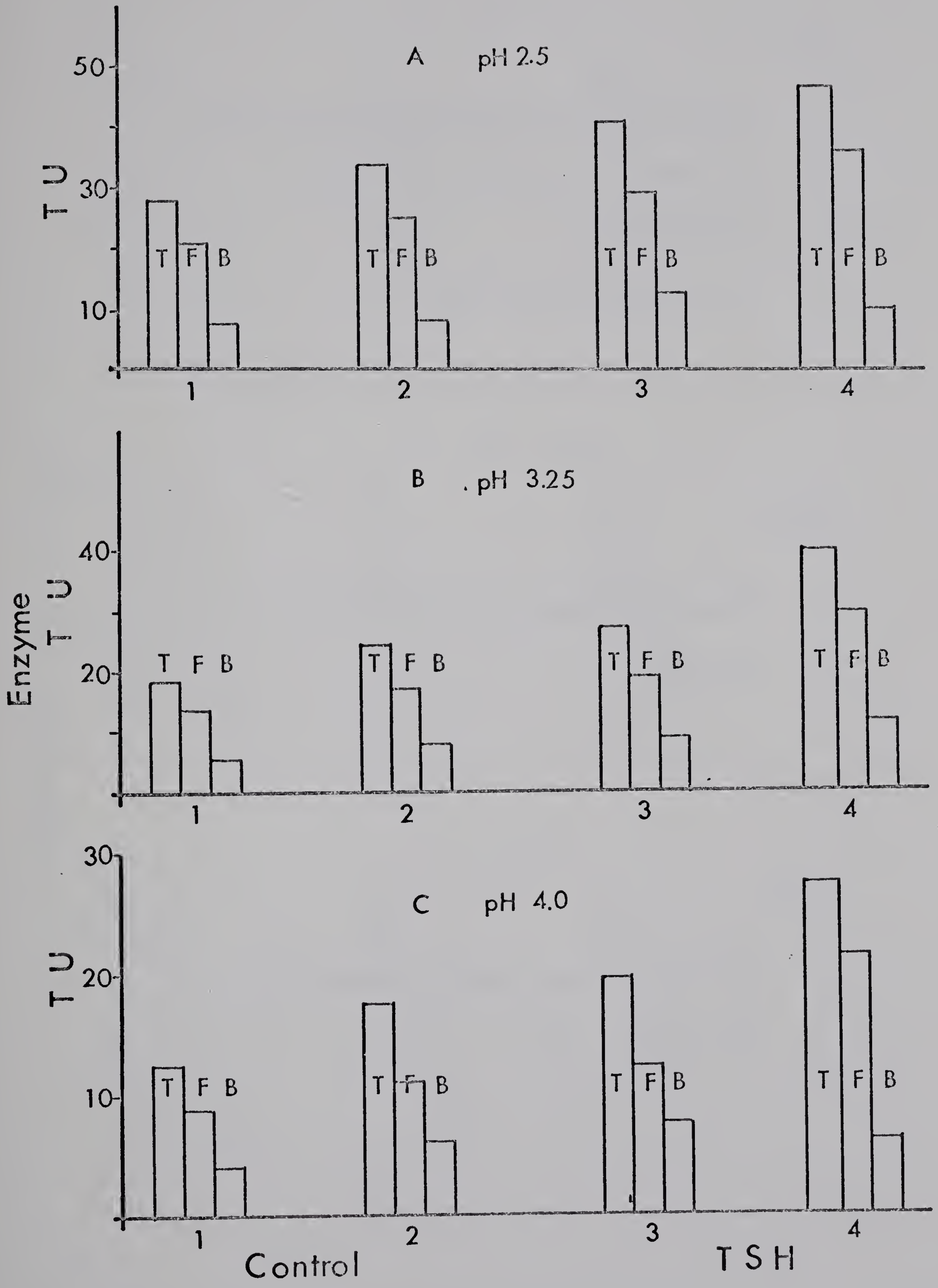

A pH 2.5
B . pH 3.25
C pH 4.0
Enzyme
T U
50
30
10
40
20
T F B
1
2
3
4
Control
T S H

Fig. 8A

The activity of free (F) and bound (B) proteinase at pH 2.5 has been expressed as a % of the total enzyme activity.

Other details are as in Fig. 7.

Fig. 8B

The same comparison is made for the activity at pH 3.25

Fig. 8C

The same comparison is made for the activity at pH 4.0

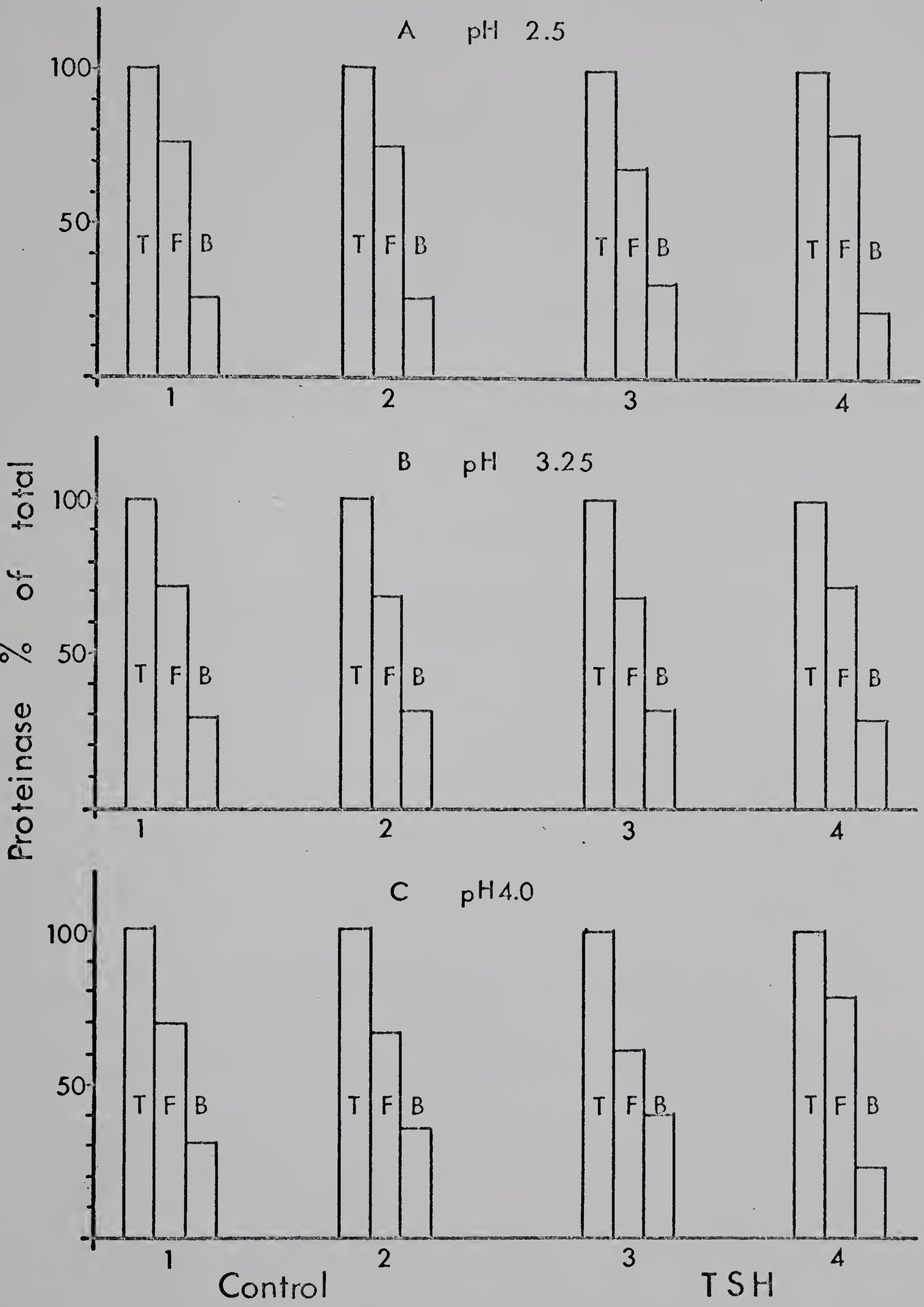

A pH 2.5
B pH 3.25
C pH4.0
100
50
T
F
B
1
2
3
4
Proteinase % of total
Control
TSH

Fig. 9A

The total activity of the enzyme at pH 3.25 and pH 4.0 has been expressed as a % of the pH 2.5 enzyme.

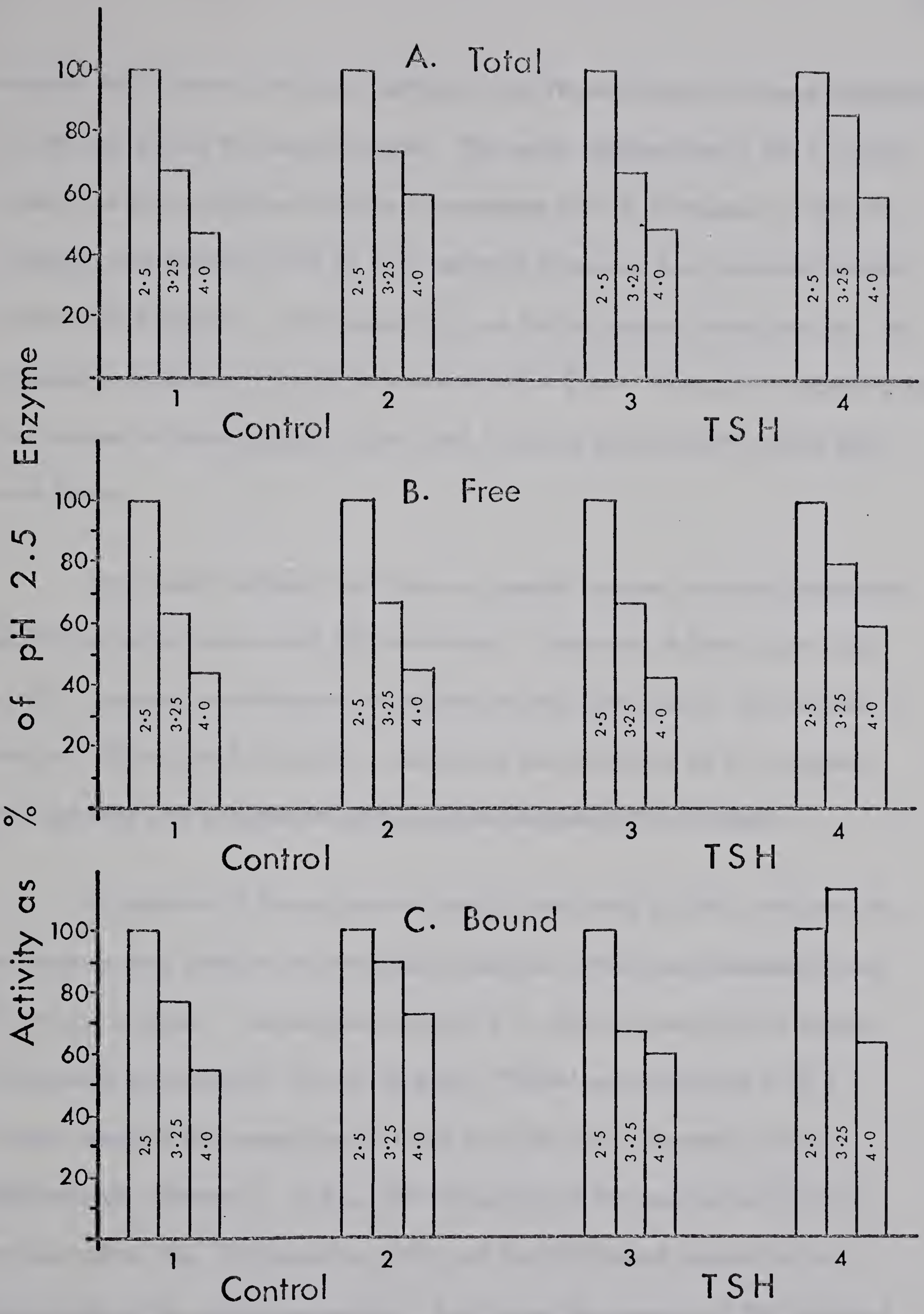
Other details are as in Fig. 7 and 8.

Fig. 9B

Activity of free enzyme at pH 3.25 and pH 4.0 has been compared to the pH 2.5 activity.

Fig. 9C

The values for bound enzyme have been subjected to the same comparison.


Activity as % of pH 2.5 Enzyme
A. Total
B. Free
C. Bound
100
80
60
40
20
2·5
3·25
4·0
1
2
3
4
Control
TSH

enzymes which make up the total activity. Fig. 9B and 9C make the same comparison for the free and for the bound fractions. The results indicate that in fish 4, which showed the most significant increase in proteinase activity in response to the TSH injection, the activity of the pH 3.25 and pH 4.0 enzymes have increased relative to the pH 2.5 enzyme. This is especially true for the lysosome bound fraction. An increase in bound enzyme activity occurred in fish 2 also. Reasons for suspecting that the increase in bound enzyme in fish 2 and 4 may not be equivalent will be dealt with below.

These results indicate that there is a general increase in thyroid proteinase activity at all pH values with TSH stimulation. The results of fish 4 suggest that a shift in enzyme from the bound to the free (active) form occurs. The increase in the pH 3.25 and pH 4.0 activities, relative to the activity at pH 2.5, suggests that enzymes with a higher pH optima may be preferentially stimulated.

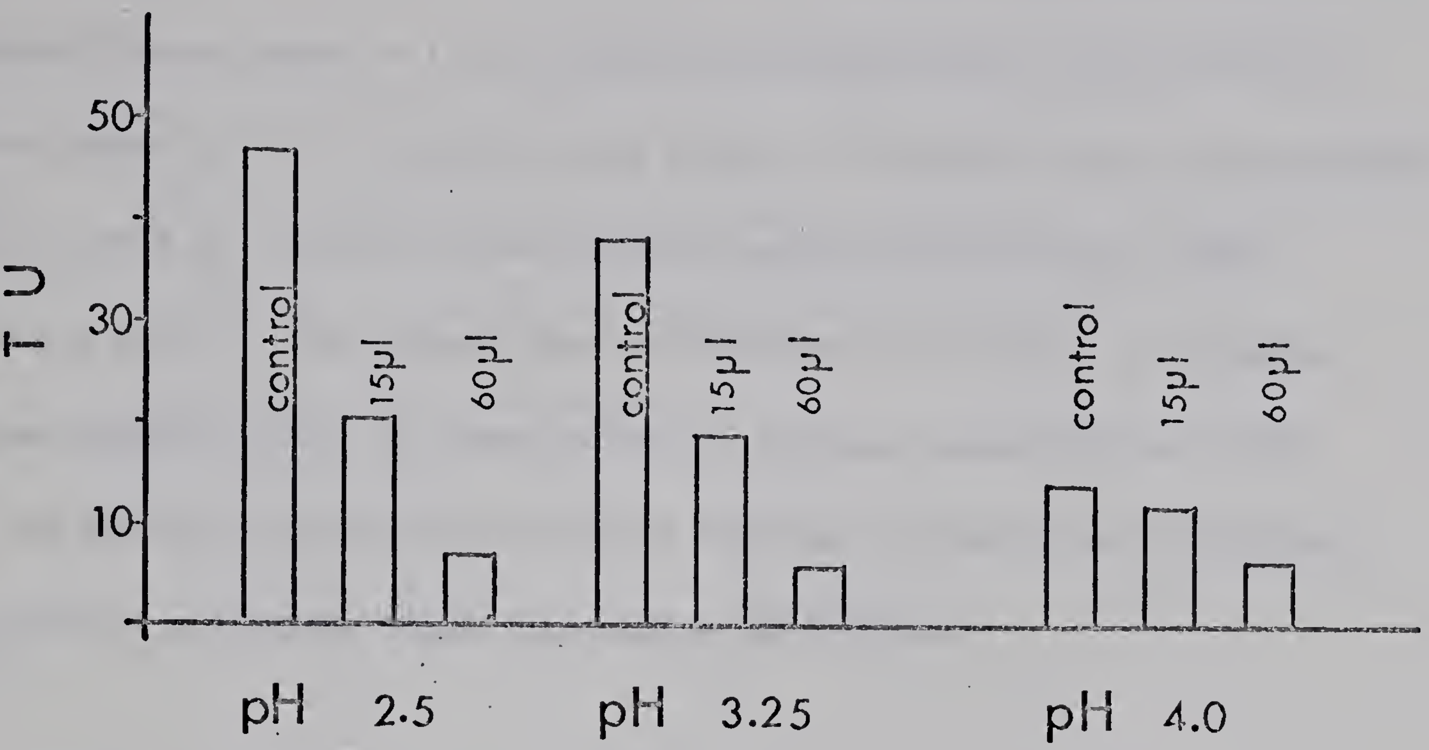
To determine if the enzyme was directly stimulated by TSH, small amounts of hormone were added to the hemoglobin substrate, which was subsequently used in enzyme analyses. The analyses of the pH 2.5, pH 3.25, and pH 4.0 enzymes on substrate containing 0, 15, and 60 μliter /TSH/ml was carried out at 25 C, using a standard TSH preparation (5 IU/ml in 0.7% NaCl, Thyrotron, Nordic Biochemicals, Montreal). In Fig. 10A the activity of the enzymes are given in tyrosine units; Fig. 10B shows the activity of the TSH treated preparation as a percentage of the control preparation. In all cases the presence of TSH inhibited the activity of the enzymes. The degree of inhibition was greatest at pH 2.5 and least at pH 4.0; at each pH the magnitude of the effect was directly proportional

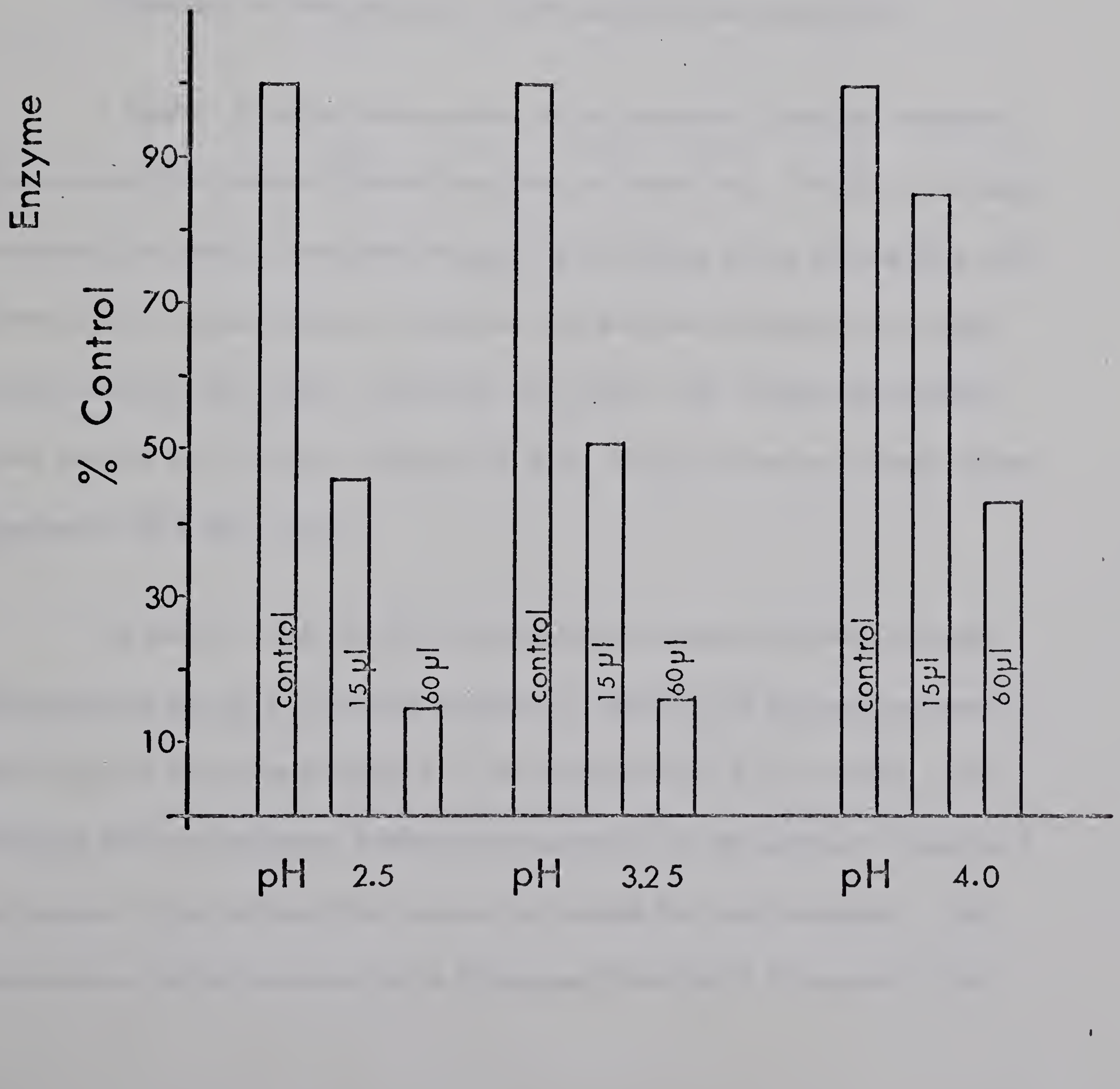
Fig. 10A

The activity of a proteinase preparation assayed at pH 2.5, pH 3.25, and pH 4.0 after the _in vitro_ addition of 0, 15, and 60 μliter of TSH. Hemoglobin substrate 30 C.

Fig. 10B

The data of Fig. 10A is presented as a % of the control value for each pH


T U
50
30
10
control
15µl
60µl
control
15µl
60µl
control
15µl
60µl
pH 2.5
pH 3.25
pH 4.0


Enzyme
% Control
90
70
50
30
10
control
15 µl
60µl
control
15 µl
60µl
control
15µl
60µl
pH 2.5
pH 3.25
pH 4.0

to the amount of hormone present. Table 5 shows the results obtained when 10 μliter of standard TSH was added to 1 ml of enzyme extract and when 10 μliter of 0.7% NaCl was added to 1 ml of control enzyme extract. When the extracts were analyzed at pH 2.5, pH 3.25, and pH 4.0 the hormone treated extract showed a slight inhibition at pH 2.5. The reduced level of inhibition in this study, as compared to the one reported in Fig. 10, seems related to the lower concentration of TSH used. The effective concentration of TSH in the final incubation medium (Table 5) was 1 μliter/ml or 1/10 the lowest dose used in the first study.

Differences in Proteinase from Warm and Cold Acclimated Fish

A number of studies were carried out to determine if enzyme extracted from summer fish thyroids differed from those of winter fish. The first experiment examined the effect of temperature upon the pH optima of the enzyme (Fig. 11). Extracts from summer fish were incubated with buffered substrate over a range of pH values at 20 C (Fig. 11A) and at 13 C (Fig. 11B). Winter fish extracts were assayed at 13 C (Fig. 11B) and 5 C (Fig. 11C). The extract remaining was assayed at 30 C (Fig. 11A).

The results of Fig. 2A (30 C) indicated the presence of three enzymes, designated as the pH 2.5 enzyme (enzyme 1), the pH 3.25 enzyme (enzyme 2), and the pH 4.0 enzyme (enzyme 3). The results of the 13 C curve (Fig. 11B) indicate that no significant differences occurred in the pH optimum of enzyme 3 or enzyme 1 when extracts from summer and winter fish were compared. Both curves show optimal values at pH 4.0 (enzyme 3) and pH 2.0 (enzyme 1) and

Table 5. Effects of TSH on the enzyme activity. The activity of thyroid proteinase at each pH was determined after the addition of 10 μliter of TSH to 1 ml enzyme.

	pH 2.5		pH 3.25		pH 4.0	
Treatment	TU	%	TU	%	TU	%
Control	43.8	100.0	30.3	100	20.3	100.0
TSH	40.8	93.2	30.3	100	19.8	97.5

Fig. 11A

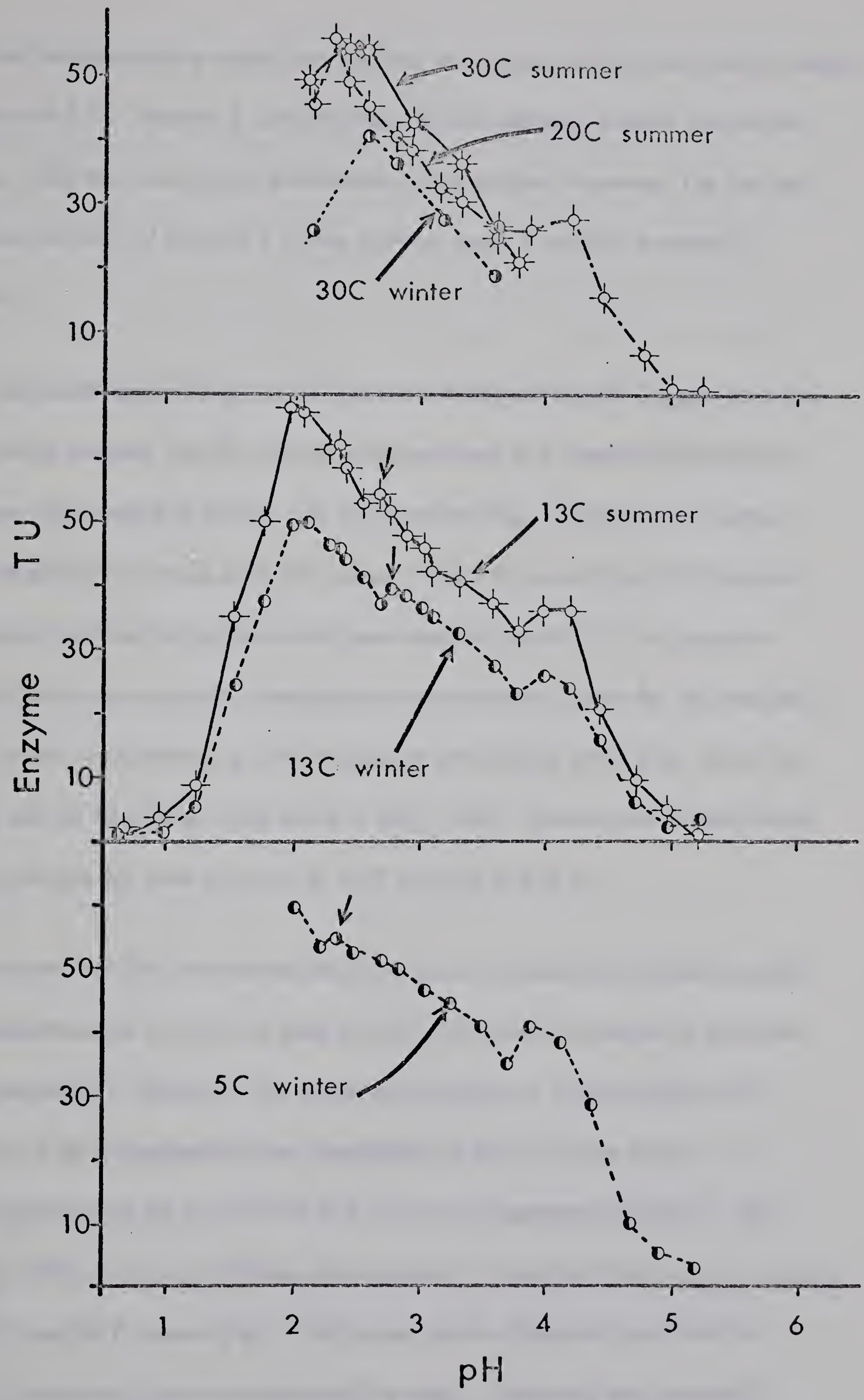
Effect of pH on activity of summer enzyme analyzed at 30 C (✱), and 20 C (⊕) and winter enzyme measured at 30 C (◑). Hemoglobin substrate.

Fig. 11B

Effect of pH on activity of summer enzyme (⊕) and winter enzyme (◑) measured at 13 C. Arrows indicate possible peak for enzyme 2.

Fig. 11C

Effect of pH on winter enzyme (◑) measured at 5 C. Arrow indicates possible peak for enzyme 2.


30C summer
20C summer
30C winter
13C summer
13C winter
5C winter
Enzyme TU
pH
10
30
50
1
2
3
4
5
6

both curves indicate that a significant decline in enzyme activity occurred at values lower than pH 2.0. Enzyme 2 activity from the two sources, summer and winter fish, (Fig. 11B) may show slight differences in pH optima; however, the low and intermittent activity of enzyme 2 in the extracts make a critical evaluation impossible.

The results obtained using the summer fish thyroid extract suggest that the optimum pH of enzyme 1 at 20 C (Fig. 11A) occurred at a lower pH than when the enzyme was assayed at 30 C. The 13 C results (Fig. 11B) showed a further shift in the optimum to more acid pH values. The 5 C curve (Fig. 11C) showed no indication that the pH optimum had been reached at pH 2.0. A change in optimal pH values as incubation temperature was reduced is seen for the enzyme 3 activity, which is evident as a distinct peak at pH 4.25 at 20 C (Fig. 11A). It is present also at 13 C (Fig. 11B) and 5 C (Fig. 11C). The enzyme 3 peak shows a shift in optimum pH from pH 4.25 at 20 C to pH 4.0 at 5 C.

Neither the 30 C curves nor the 20 C curve (summer fish thyroid extract) showed indications of an activity peak at pH 3.25 where the enzyme 2 optimum would be expected. However, the presence of enzyme 2 in both of the 13 C curves (Fig. 11B) is suggested by an irregularity at pH 2.75 (see arrow). A similar irregularity at pH 2.25 of the 5 C curve may represent enzyme 2 (see arrow, Fig. 11C). In view of these observations it is assumed that enzyme 2 occurs in the 20 C and 30 C curves (Fig. 11A) at low levels of activity and that its presence is obscured by the conditions of the assay. Assuming that enzyme 2 has been demonstrated it, like enzyme 1, shows a shift in the optimum with changes

in incubation temperature.

The rapid decline in activity seen in Fig. 11 below pH 2.0 suggests inactivation of enzymes at low pH values. Fig. 11 indicates that the effect was greater at high temperatures. The effect of pH and temperature on enzyme stability was tested by exposing portions of an extract to a low pH and neutral pH at two temperatures. Results in Table 6 show that the effect of acid pH at the higher temperature resulted in extensive enzyme inactivation. At neutral pH exposure to 30 C produced a 20% loss in activity. At 0 C pH 2.0 conditions inactivated 25% of the enzyme. The two extreme conditions combined (pH 2.0 and 30 C) produced a loss of 92% of the initial activity. Therefore, it was concluded that the apparent pH optimum at a given temperature was determined by the conditions of temperature and acidity at which inactivation of the enzyme by acid became a limiting factor.

The results presented thus far suggest that there might be seasonal differences in the thermal stability of the thyroid proteinases of the burbot. Enzyme preparations from thyroid tissue of fish caught in summer and in winter were exposed to 37 C for 20 min and then cooled in an ice bath. The results are presented in Table 7. The fish represented in Table 7A were caught 18/5/66 and 5/1/67 and analyzed 23/1/67; those in Table 7B were caught 18/5/66 and 28/1/67 and analyzed 2/2/67. The two Tables show similar results, qualitatively. The loss of activity by winter enzyme (60% in Table 7A, 40% in Table 7B) is greater than the loss by summer enzyme (11% in Table 7A, 16% in Table 7B). The pH 2.5, pH 3.25, and pH 4.0 enzymes from winter fish are all

Table 6. The effects of heat and pH on thyroid proteinase activity.

Sample	Treatment Temp. C	pH	Duration hr	Activity TU	%
1	0	7	1	36	100.0
2	30	7	1	29	80.5
3	0	2	1	27	75.0
4	30	2	1	3	8.3

Table 7. Effects of a 20 min exposure to 37 C on the activity of thyroid proteinases prepared from fish caught in summer and in winter.

Expt. A	pH 2.5		pH 3.25		pH 4.0	
	TU	%	TU	%	TU	%
winter control	53.0	100.0	33.3	100.0	12.3	100.0
winter heated	24.0	45.3	11.5	34.5	5.0	40.6
summer control	46.0	100.0	29.5	100.0	8.5	100.0
summer heated	43.0	93.5	25.6	86.8	7.3	85.9

Sources 18/5/66
5/1/66

Expt. B	pH 2.5		pH 3.25		pH 4.0	
	TU	%	TU	%	TU	%
winter control	84.5	100.0	70.0	100.0	27.5	100.0
winter heated	55.3	65.4	37.5	53.6	14.8	53.8
summer control	62.0	100.0	38.3	100.0	14.3	100.0
winter heated	58.8	94.8	32.5	84.9	10.3	72.0

Sources 18/5/66
28/1/66

more sensitive than the corresponding forms from summer fish. A slightly greater loss of activity by the pH 3.25 and the pH 4.0 enzyme is common to both experiments. A greater inactivation of the winter enzyme used in study A relative to that used in study B was noticed. Similar differences are seen in Fig. 13 and Fig. 17B. These will be dealt with in the discussion.

It is generally accepted that the Michaelis-Menten Constant (K_M), which equals the substrate concentration at which the reaction velocity equals one half the maximum velocity, is a constant characteristic of the enzyme being studied (Dixon and Webb, 1964). Michaelis-Menten Constants were determined at 30 C for the pH 2.5 proteinase extracted from tissues of summer (18/5/66) and winter (5/1/67) fish using hemoglobin as a substrate. The graphical method of Lineweaver and Burk was used to present the data in a double reciprocal plot, where 1/s is plotted against 1/v (s = substrate concentration and v = reaction velocity). The curves of Fig. 12 indicate the results. Each point is the mean of duplicate analyses. Table 8 summarizes the significant values obtained from the curve. Statistical analysis indicated that the slopes of the two lines in Fig. 12 were significantly different.

$F = 27.1$ (1-10 degrees of freedom) $> F_{.005} = 12.83$ (1-10 degrees of freedom)

One can conclude that the enzymes extracted from the thyroids of summer and winter fish are different.

The effects of temperature on the rate of enzyme activity was determined by analyzing portions of the same extract on the same hemoglobin substrate

Fig. 12

Lineweaver-Burk double reciprocal plot of the effect of substrate concentration on thyroid proteinase activity from summer and winter fish. Hemoglobin substrate pH 2.5 and 30 C.

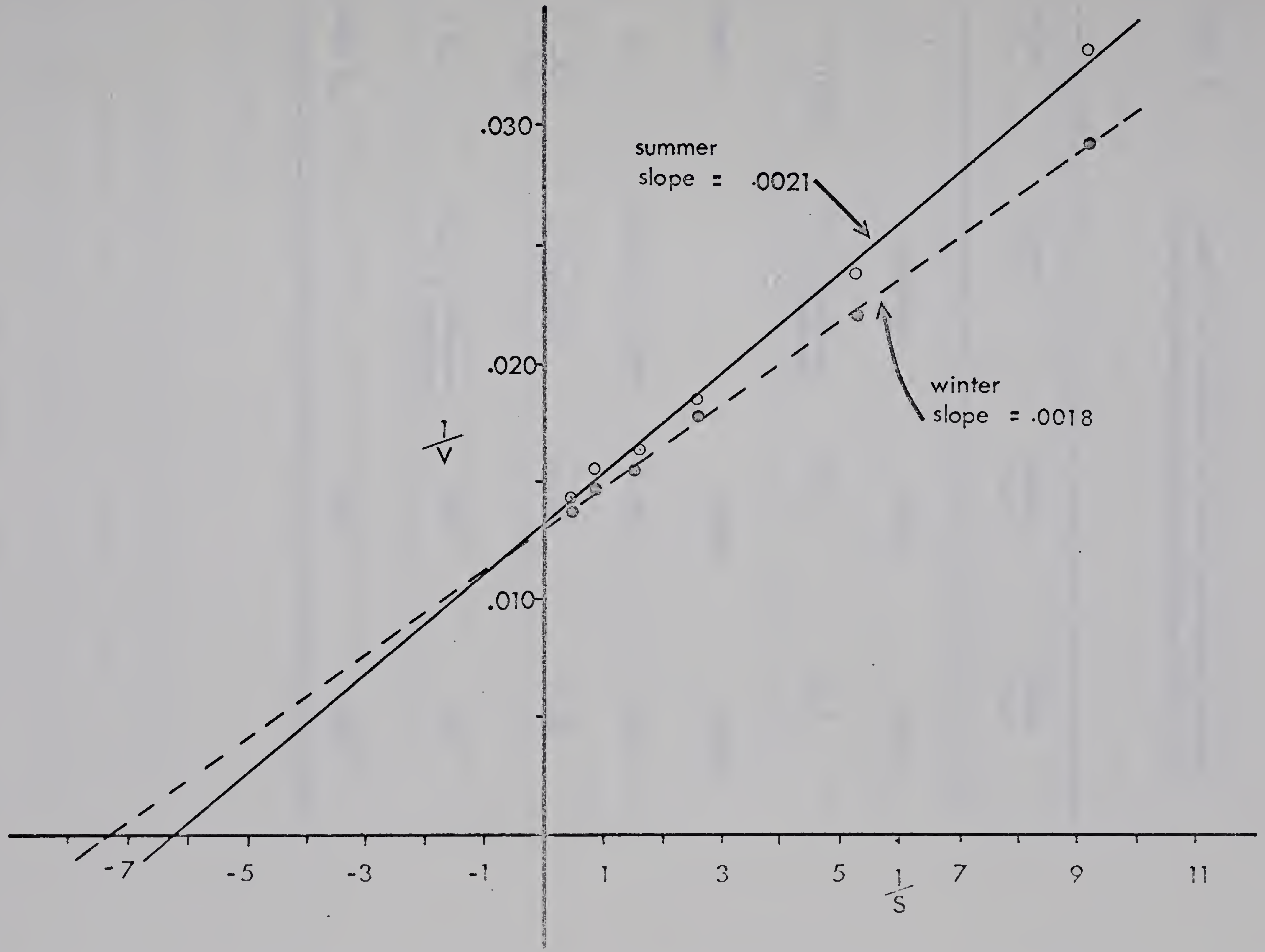

.030
.020
.010
1/V
summer
slope = .0021
winter
slope = .0018
-7
-5
-3
-1
1
3
5
7
9
11
1/S

Table 8. Summary of constants for thyroid proteinases derived from the Lineweaver-Burk analysis shown in Fig. 12.

Value	Source	Summer fish	Winter fish
$\frac{1}{V}$	y intercept	.0136	.0131
V (TU/hr)	$V = \frac{1}{\text{y intercept}}$	73.4	76.2
Slope		.00209	.00179
K_m	$K_m = V \cdot \text{slope}$	.153	.136
$-\frac{1}{K_m}$	x intercept	-6.45	-7.30
K_m	$K_m = \frac{1}{\text{x intercept}}$	.155	.137
mean K_m		.154	.137

preparation at a series of temperatures. The work was done in two parts; on 13/4/67 extracts were prepared representing summer fish (16/7/66) and winter fish (28/2/67). These were analyzed in duplicate at 0, 5, 10, 15, and 20 C. On 10/5/67 new extracts were made from winter fish tissue (5/1/67) and from summer fish tissue (18/5/66). These were analyzed in quadruplicate at 5, 10, 15, and 20 C. The mean values were graphed as a simple rate-temperature curve (Fig. 13A) and as an Arrhenius plot (Fig. 13B). In Fig. 13A the Q_{10} of the reaction is given by the slope of the line while in Fig. 13B the slope is proportional to μ, the critical thermal increment (apparent activation energy).

The curves for February (2), May (3), and July (4) show a two phase relationship which was not seen in the January (1) curve (Fig. 13A and 13B). The critical temperature (change in slope indicated by arrow) is about 10 C for the February curve, 12 C for the May curve and at about 15 C for the July curve. The Q_{10} values calculated for 5 C increments are presented in Table 9. In general, the Q_{10} values for enzyme activity from winter fish are higher than those for activity from summer fish, measured over the same temperature range.

Two preliminary studies were done to determine how exposure of fish to cold affected the activity of thyroid proteinase. In the first study the effects of a rapid transfer of the fish from 15 C to 5 C on the activity of the pH 2.5 proteinase (hemoglobin substrate) was tested. The activities of the thyroid proteinase after a 12 hr exposure to the lower temperature are

Fig. 13A

The rate-temperature curve for enzymes prepared from burbot thyroids collected 5/1/67, 28/2/67, 18/5/66, 16/7/66 as measured on hemoglobin substrate. The units of activity are arbitrarily chosen so that the curves occur in a chronological sequence according to month. The arrows indicate the critical temperature. Values for July and Feb. curves are means of duplicate analyses. Values for Jan. and May represent means of quadruplicate analyses. Analyzed at pH 2.5 and 30 C.

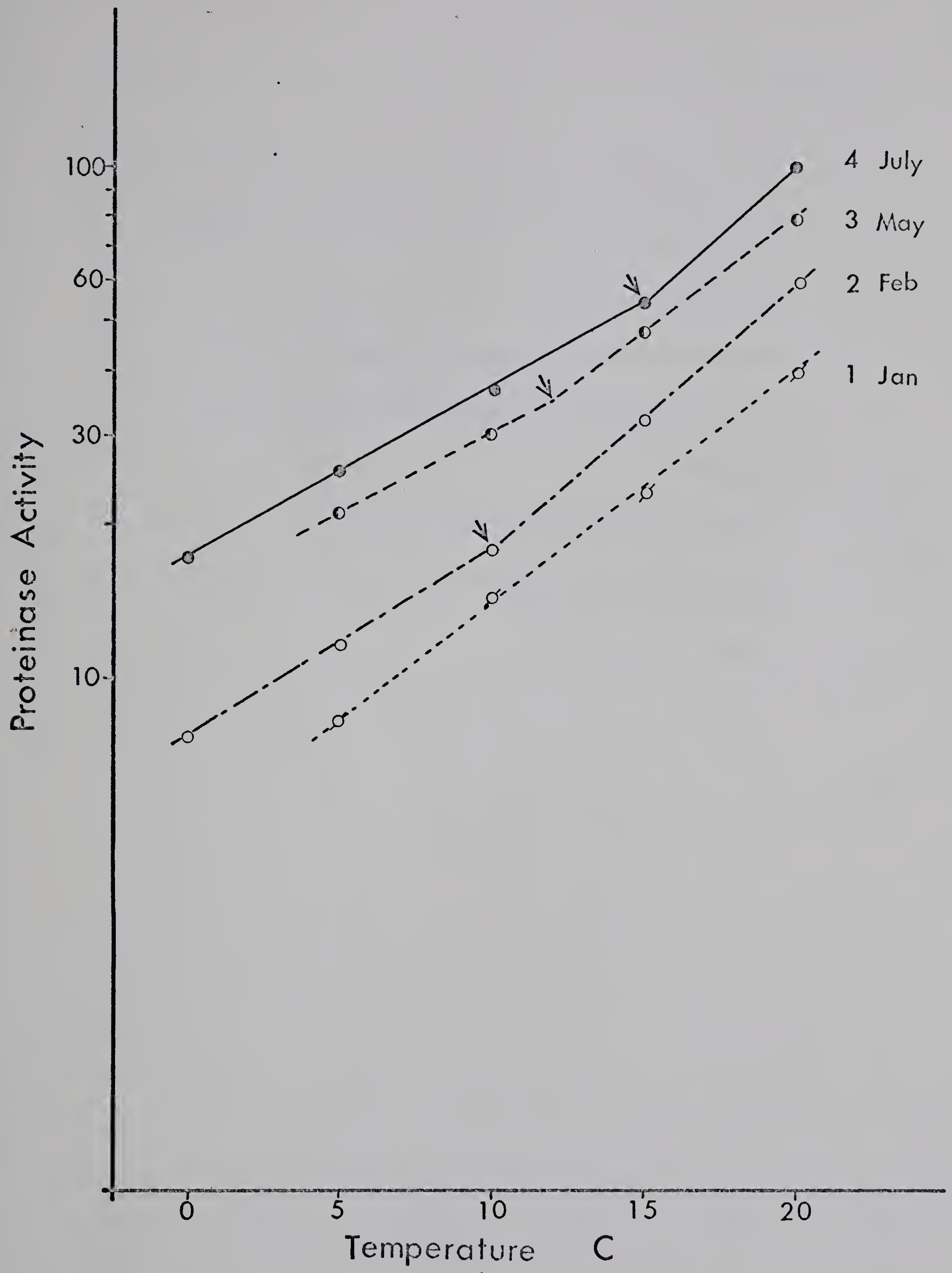

100
60
30
10
Proteinase Activity
0
5
10
15
20
Temperature C
4 July
3 May
2 Feb
1 Jan

Fig. 13B

Arrhenus plot of enzyme activity against reciprocal of absolute temperature for enzymes from 5/1/67, 28/2/67, 18/5/66, and 16/7/66.

The units of activity are arbitrary, chosen so that the curves would occur in a chronological order according to month.

The arrows indicate the critical temperature.

Other details are as given in Fig. 13A

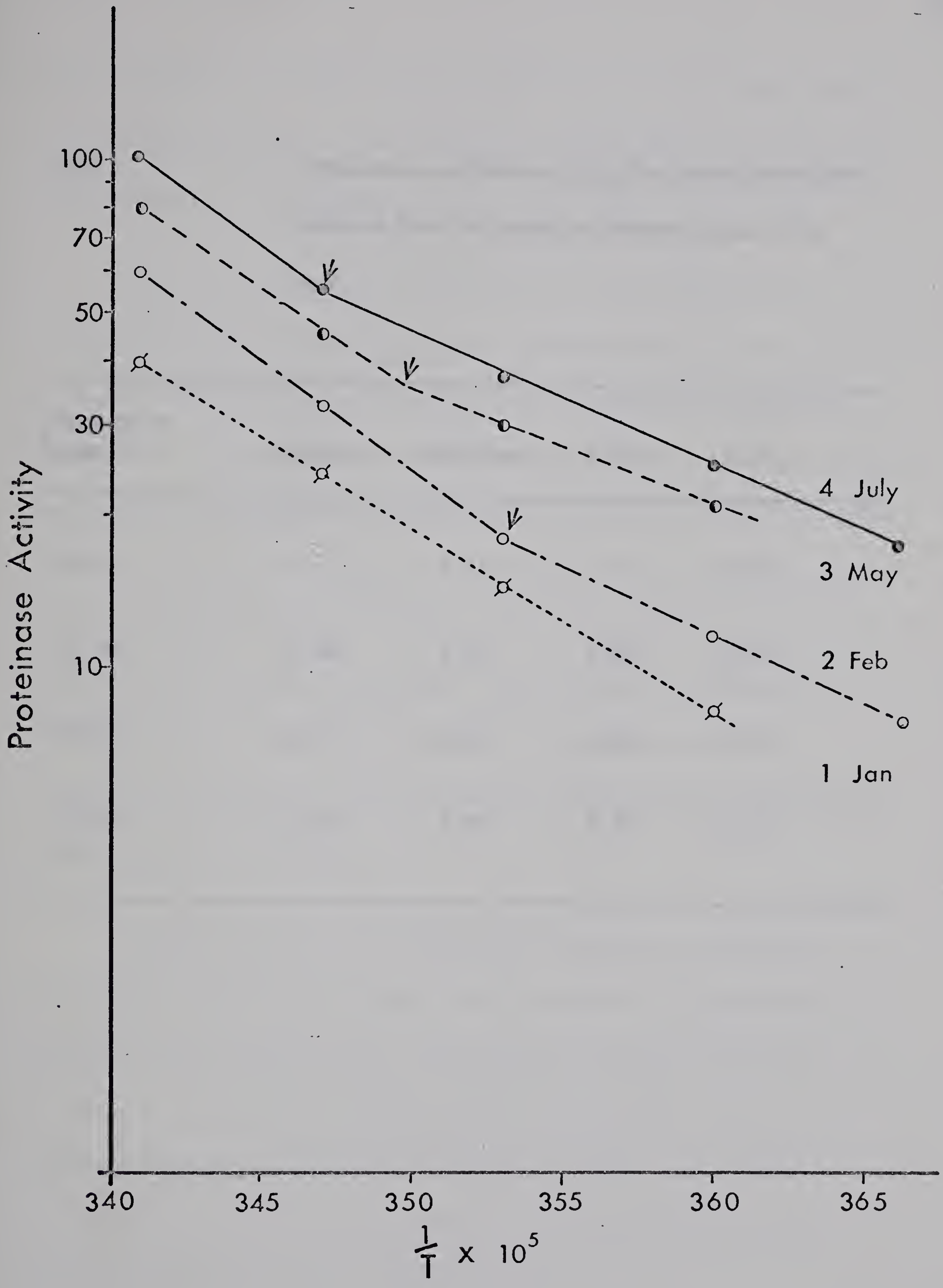

Proteinase Activity
100
70
50
30
10
340
345
350
355
360
365
$\frac{1}{T} \times 10^5$
4 July
3 May
2 Feb
1 Jan

Table 9. Temperature coefficients (Q_{10}) for thyroid proteinases prepared from fish caught at different times of the year.

Temperature Range C	5 January	28 February	18 May	16 July
0-5	-	2.16	-	2.08
5-10	3.06	2.37	2.03	2.04
10-15	2.61	3.63	2.53	2.39
15-20	2.93	3.45	2.79	3.37

shown in Table 10. The Mann Whitney U test (Siegal, 1956) indicates that the differences are significant at the 0.10 level, indicating that the enzyme levels were reduced by cold exposure.

Fig. 14 shows the effects of acclimation to 4 C by summer fish as compared to control fish caught at the same time and held at 12 C to 17 C for 6 weeks. Because of the limited sample size and the extreme variability of the results the data were not analyzed statistically. Only spleen enzyme showed any evidence of low temperature compensation. Enzyme activities from thyroid, kidney, conus, and muscle appeared unaltered while the activity of the liver enzyme was lower in the low temperature group. The significant feature is that the "same" enzyme(s) from different organs analyzed on hemoglobin at pH 2.5 may show increased, decreased, or no change in activity during temperature acclimation. There is, of course, no proof that the enzymes from different organs are the same.

It appears likely that liver (and possible muscle) are responding to the nutritional state of the fish rather than to temperature. These fish had been held without food for several weeks and were probably utilizing liver reserves and muscle protein as energy sources (Hoar, 1957). This would be especially important at high temperatures (Brown, 1957). Umana (1967) has shown that starvation of rats increases the quantity of liver cathepsins which are presumably involved in gluconeogenesis.

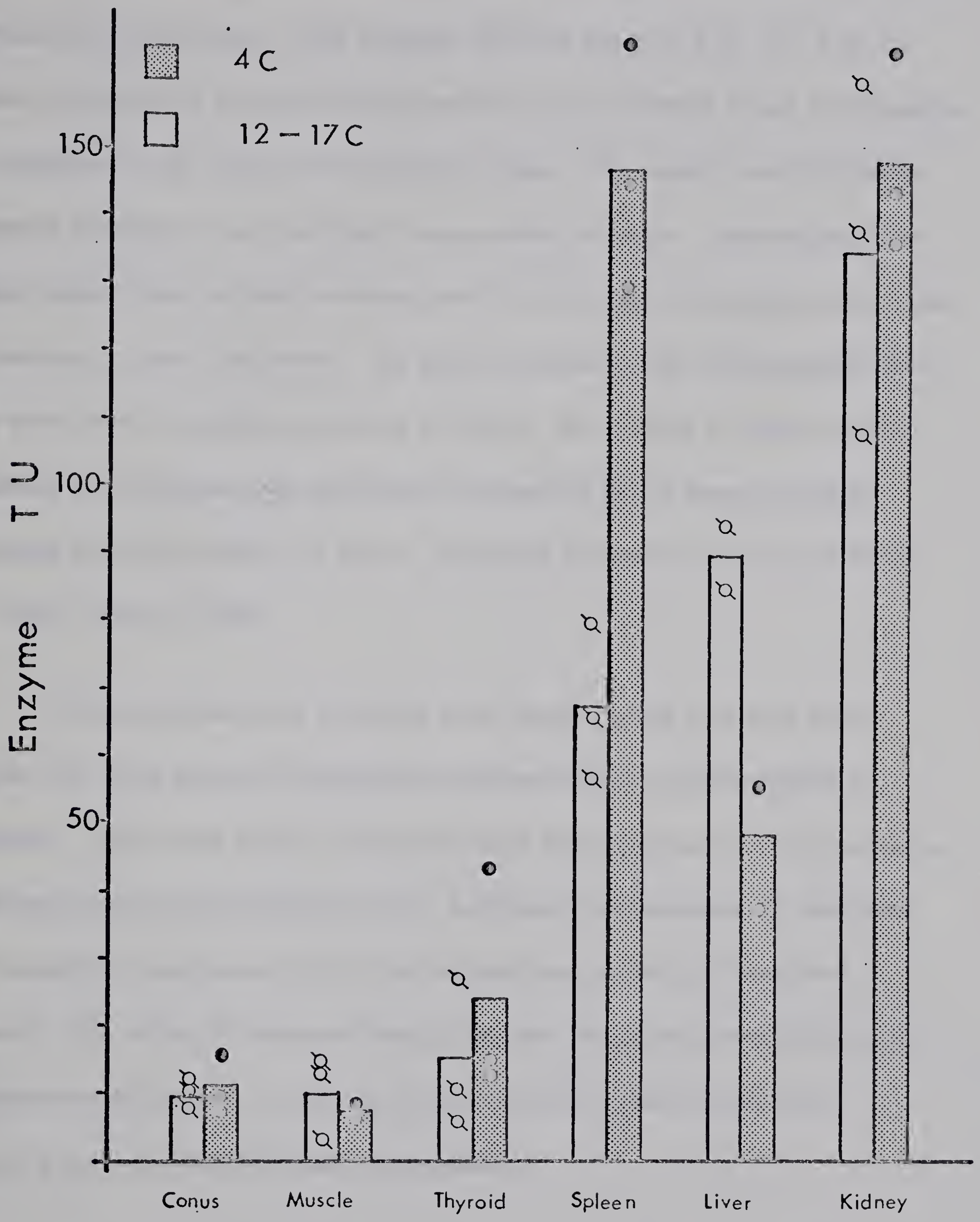
Two factors pertinent to this study that show obvious seasonal variation

Table 10. Effect of 12 hr cold shock; transfer from 15 C to 5 C. on the thyroid proteinase of burbot.

Treatment	Temp. C	Sample size	enzyme activity mean TU	enzyme activity range TU
control	15	4	27.8	14.3-43.8
cold shock 12 hr, 10 C below acclimation temperature	5	4	16.6	10.1-21.0

Fig. 14

Activity of enzyme extracted from different tissues after acclimation of burbot to high temperature (12 to 17 C) and low temperature (4 C). Assayed in duplicate on hemoglobin at pH 2.5. The bars are drawn to represent the mean values while the points indicate the values for individual fish which are means of duplicate determinations at 25 C.


4 C
12 – 17 C
150
100
50
Enzyme T U
Conus
Muscle
Thyroid
Spleen
Liver
Kidney

are environmental temperature and reproductive activities of the burbot. Seasonal temperatures from January 1966 to March 1967 are shown in Fig. 15. Fig. 16 shows the pattern of gonadal development for male and female burbot which begins in September as the lake water temperature drops. The smooth curve for female gonadal development was sketched to approximate the data. Maturation of the testes appears more variable but more rapid than ovarian development and reaches a maximum by about December. The ovary increases in size more gradually than the testes until it reaches a maximum in March. The decline in testes weight in February may indicate early spawning; the observed loss of sperm caused by handling the fish probably is a factor. Spawning occurred in mid to late March in 1965, 1966, and 1967.

Thyroid samples were collected at all seasons of the year from burbot in Lac Ste. Anne and were analyzed for enzyme activity using hemoglobin substrate. Each value in Fig. 17 represents the mean enzyme activity in extracts of thyroid tissue from six different fish. Each assay was performed in duplicate on hemoglobin substrate at pH 2.5 and in some cases at pH 3.25 and pH 4.0 as well. The values for male and female fish were examined and the differences appeared to be random. It was concluded, therefore, that no consistent sexually related effects had been demonstrated.

During the fall when lake temperature was dropping, enzyme activity appeared to remain constant at a moderately high level. Later in the winter the activity of the thyroid enzyme had fallen by about 30% (statistically significant, P = .05, Table 11) and remained low until mid May. Enzyme

Fig. 15

Seasonal variation in water temperature at Lac Ste. Anne measured at a depth of 3 m (from May 1966 to April 1967)

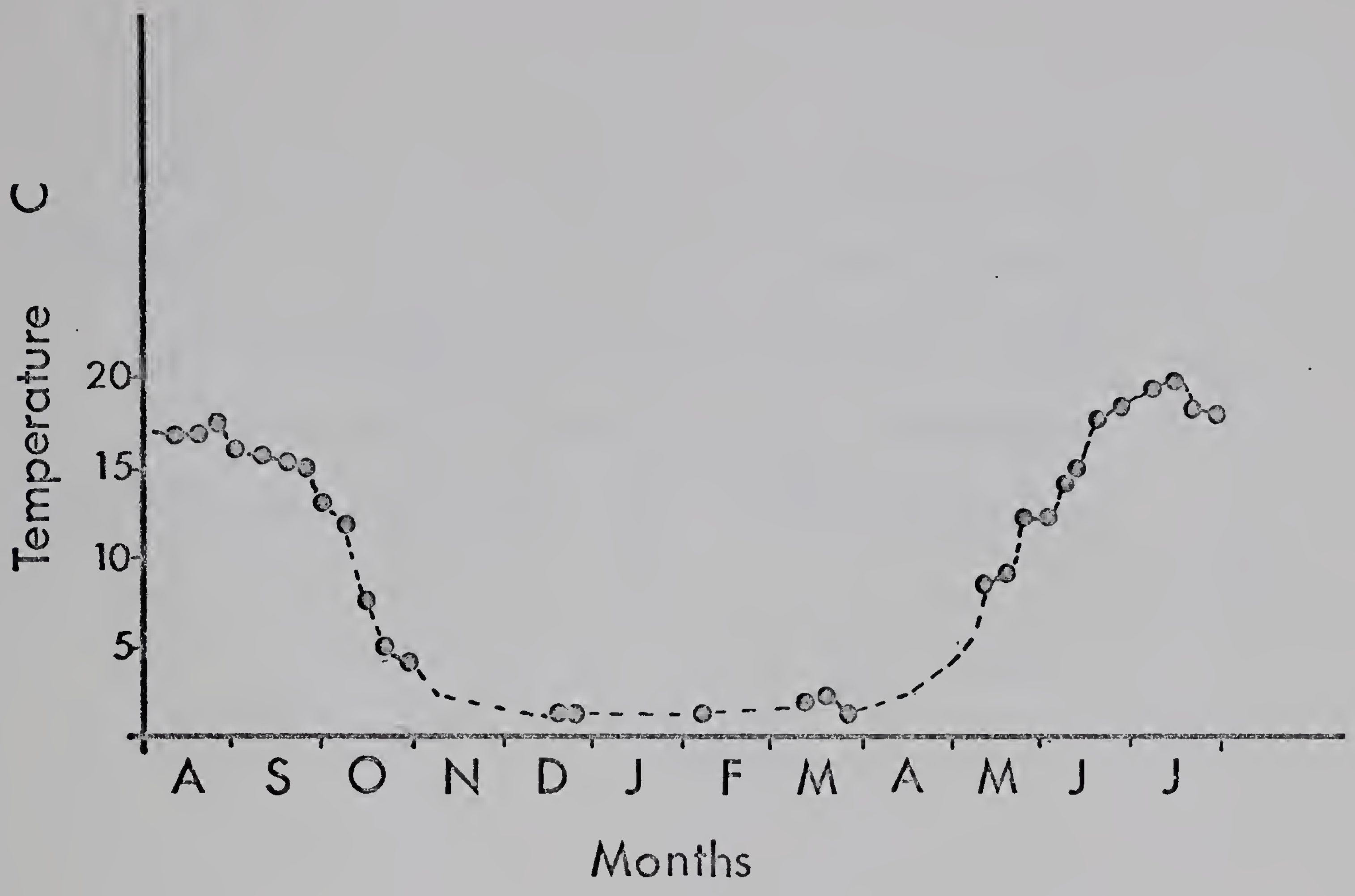
Fig. 16

Seasonal change in gonadosomatic index (GSI) of male and female burbot collected from Lac Ste. Anne, Alberta.

$$\mathrm{GSI} = \frac{\text{gonad weight}}{\text{body weight}} \times 100\%$$

♂ = ●

♀ = ○


Temperature C
20
15
10
5
A S O N D J F M A M J J
Months

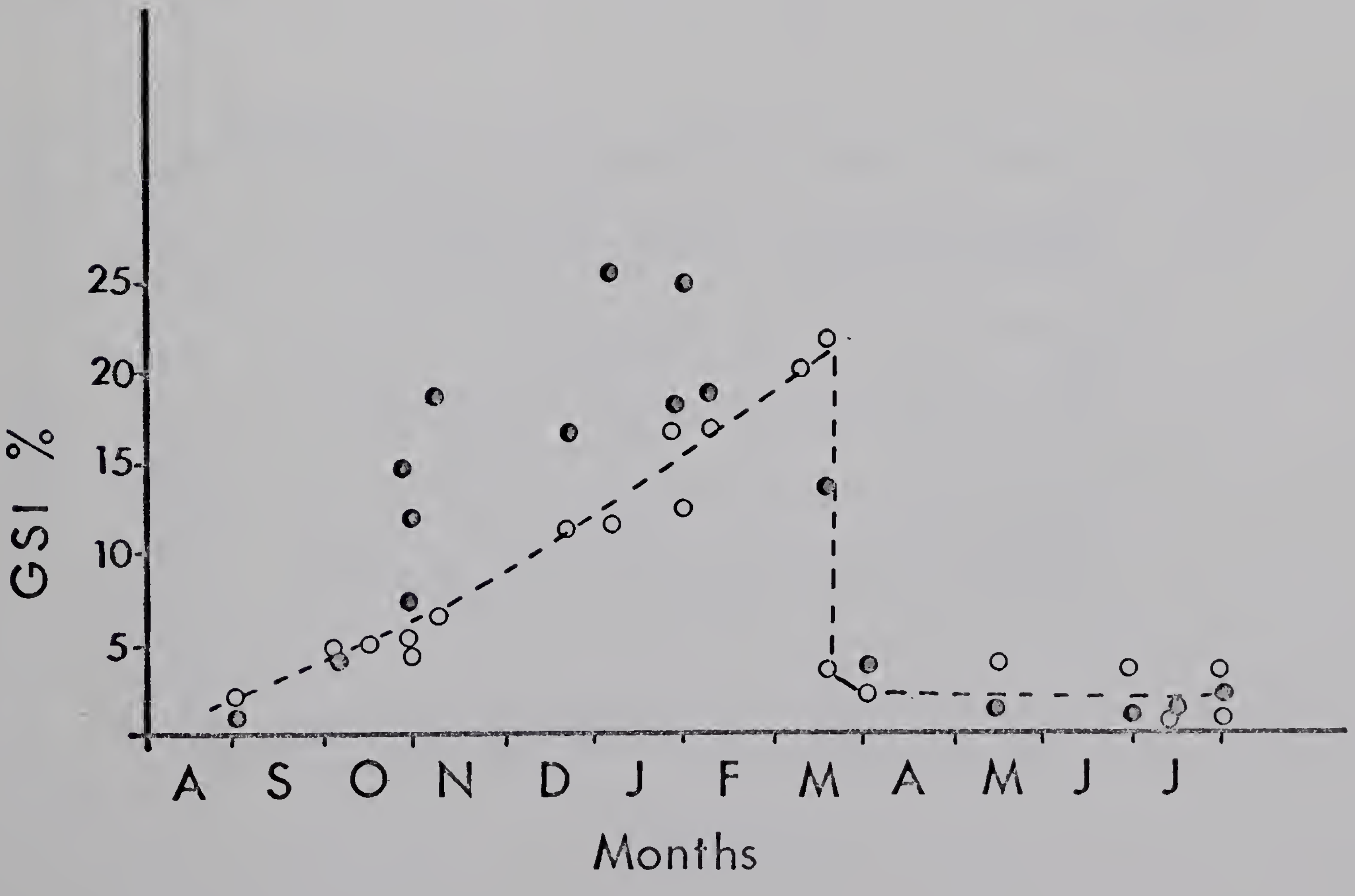
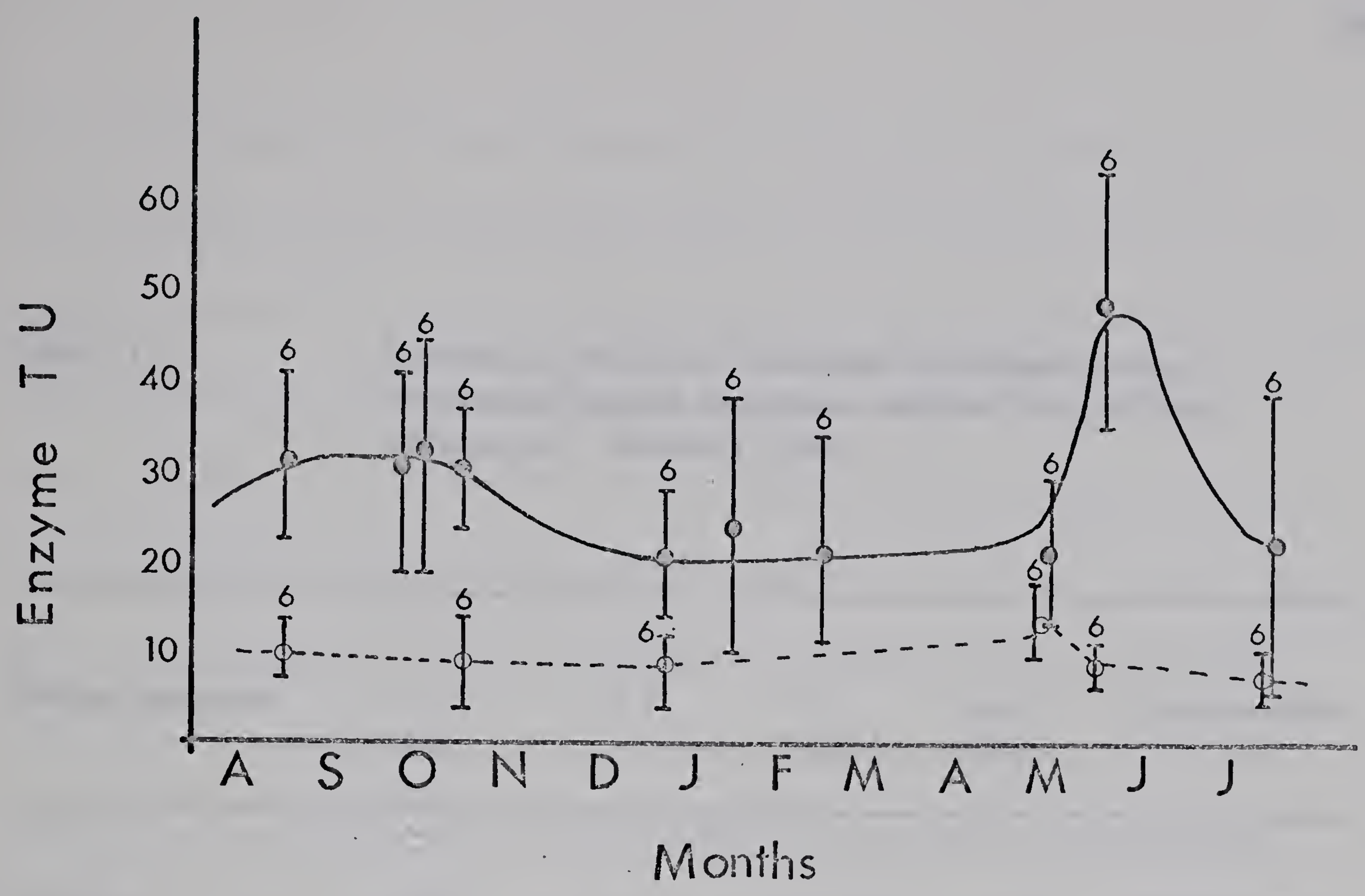

GSI %
25
20
15
10
5
A S O N D J F M A M J J
Months

Fig. 17A

Seasonal changes in pH 2.5 thyroid proteinase (●) activity of burbot collected at Lac Ste. Anne and assayed on hemoglobin at 25 C. Points are means of duplicate determinations on 6 individual fish. Similar analyses of extracts from conus arteriosis are reported as (○). Bars equal one standard deviation.

Fig. 17B

Seasonal change of thyroid proteinase activity of burbot at pH 3.25 (curve A – ∅) and pH 4.0 (curve C – ○) relative to pH 2.5 activity. Change of pH 4.0 (curve B – ✱) activity relative to pH 5 activity. In each case the mean activity at each pH (duplicate values for 6 fish) has been used in calculating the ratio.


Enzyme T U
60
50
40
30
20
10
6
A S O N D J F M A M J J
Months

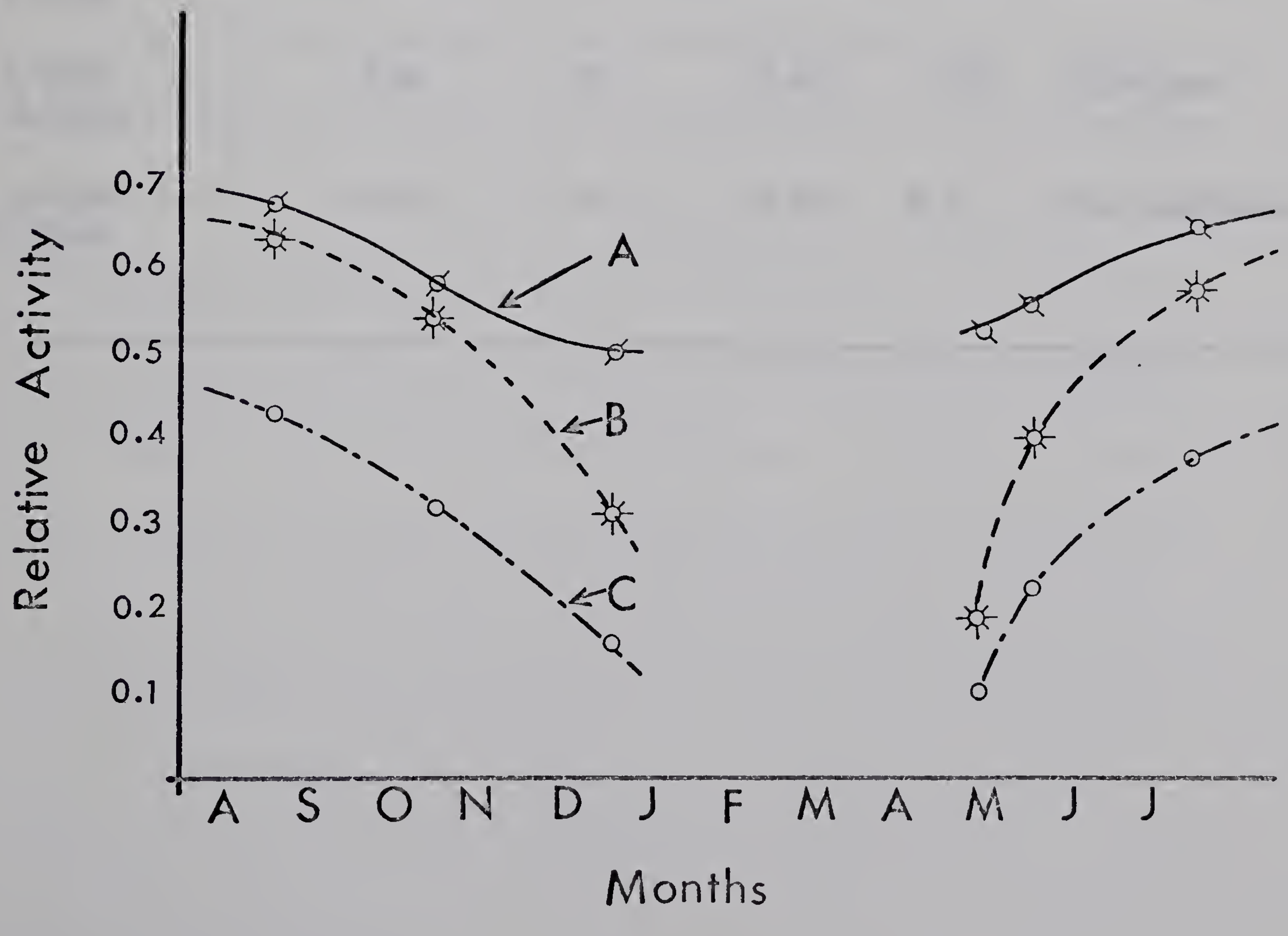

Relative Activity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A
B
C
A S O N D J F M A M J J
Months

Table 11. Summary of statistical tests used to compare means of seasonal thyroid proteinase samples for significant differences. Student's t-test.

Groups compared	t calc.	d.f.	t tabled	level of sig.	interpretation
29/10/66 5/1/67	2.22	10	2.23	.05	Significant
18/5/66 2/6/66	4.06	10	3.58	.005	Significant
2/6/66 30/7/66	3.08	10	2.63	.025	Significant
30/7/66 2/9/66	1.19	10	0.88	0.4	Not significant

activity from burbot collected 2 June had risen sharply to a concentration 2 times the mid winter value (statistically significant, P = .005, Table 11). This increase paralleled the increase in water temperature during May and June. Enzyme activity then declined (statistically significant, P = .025, Table 11) and values obtained at the end of July were the same as those found in mid winter. Extracts of conus arteriosis tissue were analyzed for comparison. When the study was begun the conus arteriosis was selected as a control tissue because of its proximity and similarity to the ventral aorta. It was realized later that significant amounts of thyroid tissue may be found in the conus (Baker-Cohen, 1959). For this reason additional analyses of control tissue was not done. The activity of the enzyme in the conus arteriosis remained consistently low throughout the year.

Fig. 17B shows the mean activity of the pH 3.25 and pH 4.0 thyroid proteinase expressed as a fraction of the proteinase activity at pH 2.5 (lines A and C). The value of the mean pH 4.0 enzyme activity is expressed as a fraction of the mean pH 3.25 activity (line B). The ratios plotted on a seasonal basis demonstrate that the relationship between the activities at different pH values is not constant. The activities of the pH 3.25 enzyme and the pH 4.0 enzyme, relative to the pH 2.5 enzyme, are seen to decline in the fall and increase in the spring. The pH 4.0 enzyme shows a much greater change seasonally than the pH 3.25 enzyme. The ratio of the pH 4.0 enzyme to the pH 3.25 enzyme showed a similar pattern. This is strong evidence that there is a seasonal change in the relative activities of the different enzymes independent of changes in their absolute activities.

After the analysis of individual extracts the enzyme extracts were pooled. Portions of the pooled extracts from each collection period were used to determine whether seasonal differences in inactivation temperature, response to cysteine, and response to iodoacetate were evident. The methods used have been described above. Table 12 shows the results of these analyses which were carried out in duplicate with hemoglobin substrate at pH 2.5. The cysteine results showed no significant trends but the iodoacetate treatment showed a seasonal pattern with enzymes from winter fish exhibiting a greater degree of inhibition. The results of the temperature inactivation study confirmed the earlier observation (Table 7) that enzyme from fall and winter was more sensitive to heat than enzyme prepared from summer fish tissue.

Iodide Distribution Studies

Normal thyroid function is dependent upon an adequate supply of iodide. A study of iodide metabolism in the burbot was undertaken to determine to what extent the pattern of iodide distribution and seasonal fluctuations in iodide concentration might influence thyroid activity.

In the first study radioiodide was injected into a group of fish, from which individuals were sampled at intervals of time after injection. Weighed tissue samples were counted for radioactivity and changes in the distribution of the isotope determined by calculating tissue/serum (T/S) ratios of radioiodide. The results for tissues representing different patterns of iodide distribution are presented in Fig. 18 and Fig. 19.

Table 12. Effects of exposure to heat, cysteine, and iodoacetate on enzymes collected at different times of the year.

Date	Control		T inact.		Cysteine		Iodoacetate	
	TU	%	TU	%	TU	%	TU	%
2/9/66	16.7	100	15.0	90.1	21.0	126.0	9.2	55.0
9/10/66	12.5	100	9.1	72.7	26.0	209.0	2.8	22.5
5/1/67	14.4	100	10.9	76.0	21.6	151.0	2.2	15.0
18/5/66	14.9	100	11.8	78.9	28.6	192.0	1.75	11.7
2/6/66	19.7	100	18.0	91.1	25.8	131.0	2.85	14.5
30/7/66	10.2	100	9.3	90.7	18.9	185.0	5.05	49.5

Fig. 18

Changes in the T/S ratios of skin (⌀), bile (◓) and muscle (○) after the injection into burbot of a tracer dose of radioiodide. Lines are drawn to indicate trends.

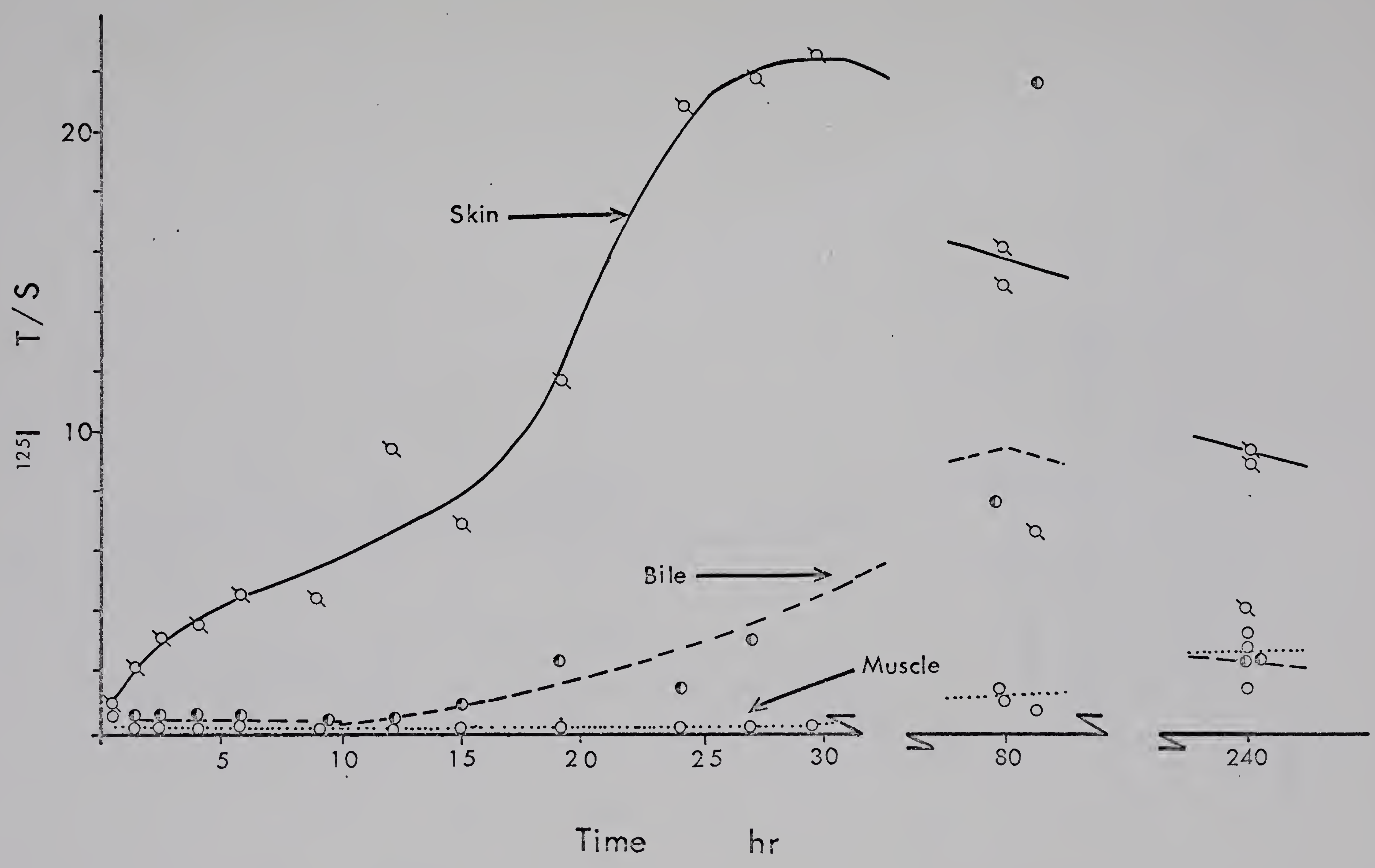
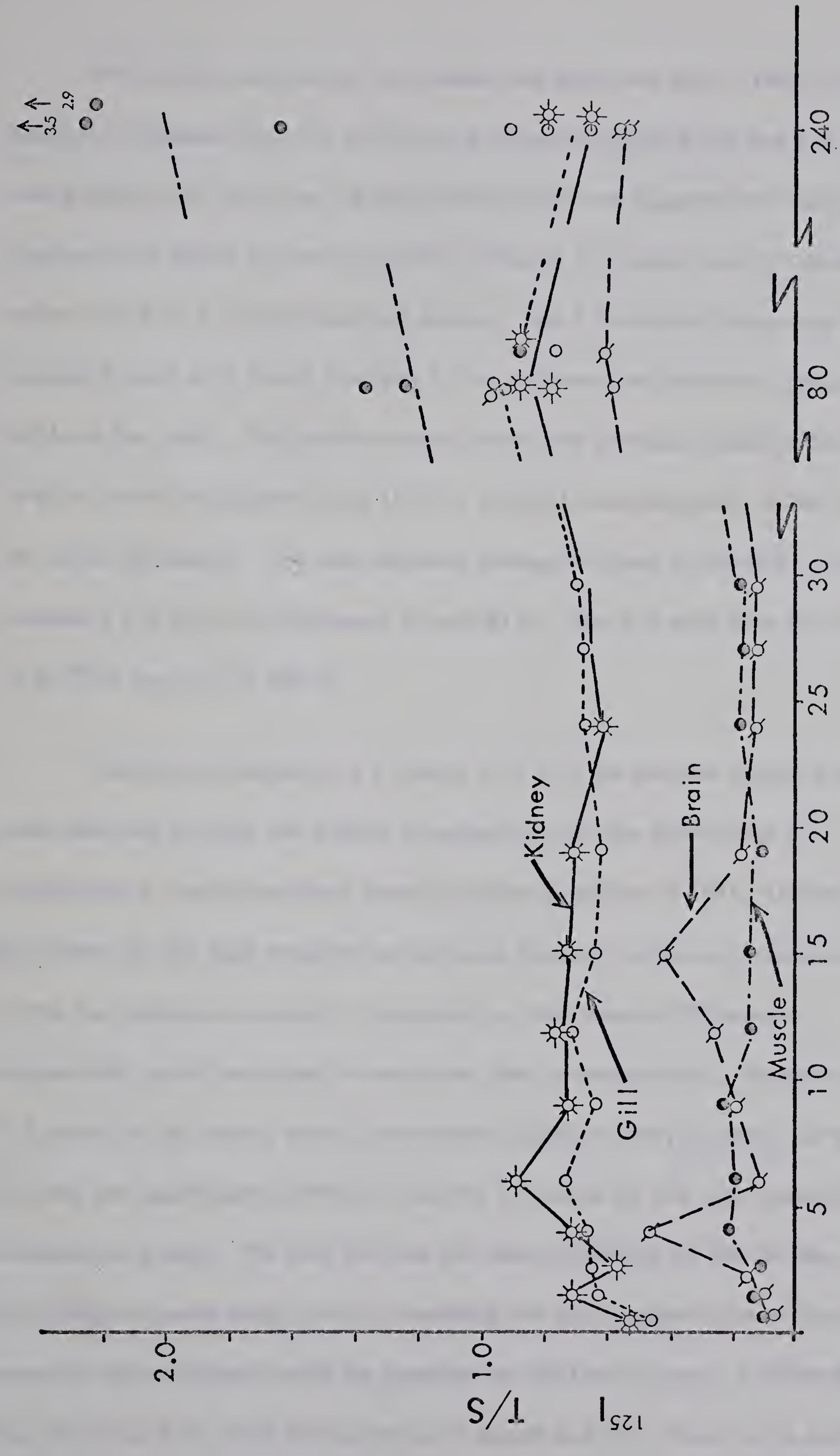

20
10
125I T/S
Skin
Bile
Muscle
5
10
15
20
25
30
80
240
Time hr

Fig. 19

Changes in the T/S ratio of muscle (●), brain (ϕ), gill (○), and kidney (⊛) after the injection into burbot of a tracer dose of radioiodide. Lines are drawn to indicate trends.


^{125}I T/S
2.0
1.0
Time hr
5
10
15
20
25
30
80
240
Kidney
Gill
Brain
Muscle
3.5
2.9

Radioiodide penetration into muscle and brain was slow. The T/S ratio gradually increased from 0.2 at 24 hr to a value of 0.6 for brain and 0.8 for muscle 80 hr after injection. Brain iodide values then appeared to remain constant until 240 hr but muscle iodide continued to increase until it attained values of 1.6 to 3.3 in the terminal sample. The T/S ratio of kidney and gill reached a level of 0.6 to 0.8 within 2.5 hr and remained relatively constant for the 240 hr of the study. The iodide content of the bile increased steadily from 15 hr to 80 hr, when its highest values (T/S = 8 to 22) were recorded. After this time the values decreased. The most dramatic change was seen in the skin, which reached a T/S ratio of 22 between 25 and 30 hr. The T/S ratio then fell to about 11 at 72 hr and to 7 at 240 hr.

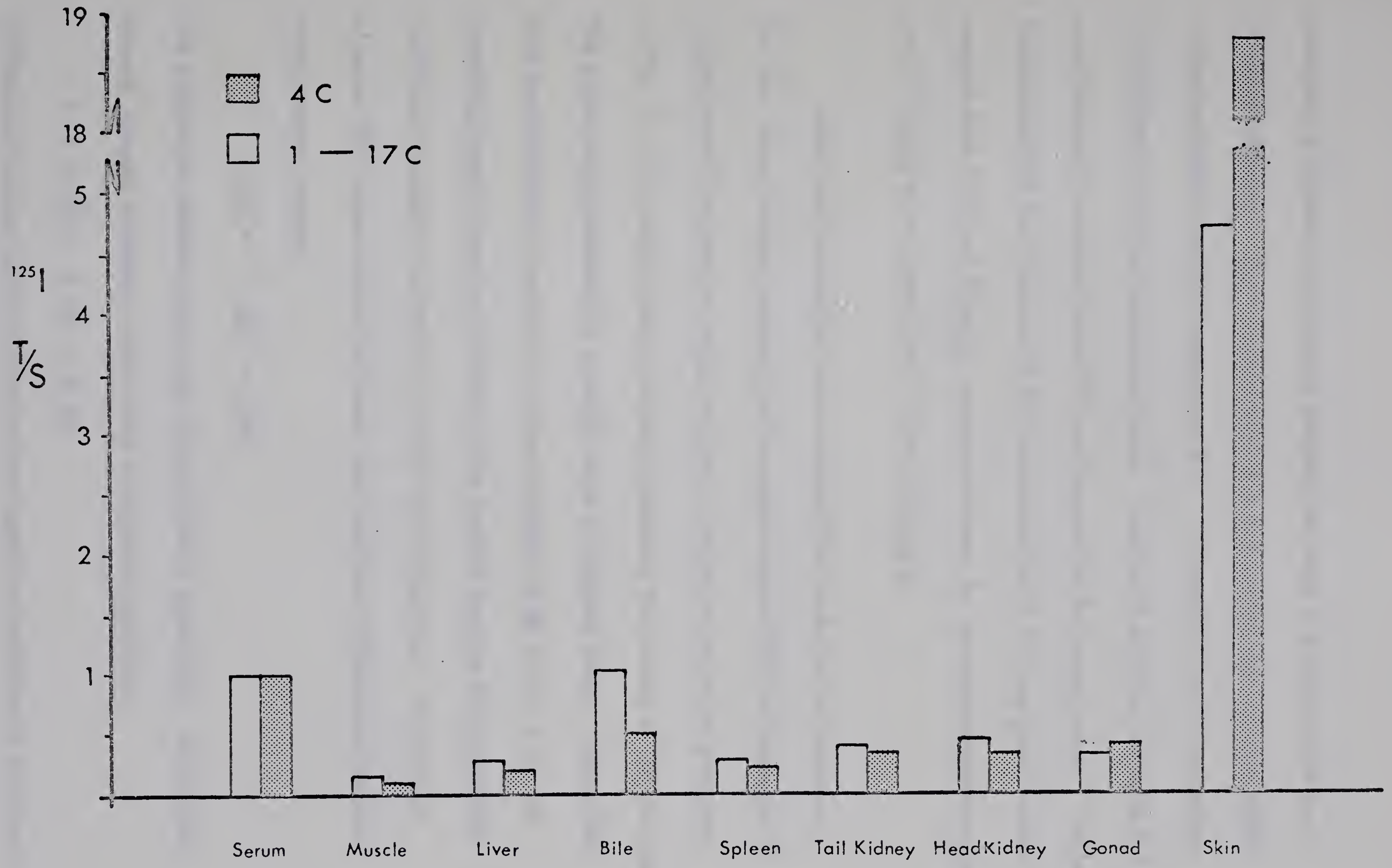
The fish acclimated to 4 C and to 12 - 17 C for enzyme studies (Fig. 14) were used also to study the effects of temperature on the distribution of radioiodide in the tissues taken three days after injection of ^{125}I. Unfortunately, by chance all the high temperature fish were females, while only males occurred in the low temperature group. Consequently, the observed differences conceivably could be related to sex rather than to temperature. However, the T/S ratios for the ovary, which concentrates iodide in many animals, were low and not significantly different from the T/S ratios for the male gonad (low temperature group). The fact that the fish were studied at the end of May (27/5/66) and were either spent or resorbing the ova, suggests that minimal sexually related effects would be expected at this time of year. Reference to Fig. 20 shows that, with the exception of gonad and skin, there was a small

Fig. 20

Radioiodide T/S ratios of different tissues from warm (12-17 C) and cold (4 C) acclimated burbot.

Duration of acclimation 6 weeks.

Fish sampled 72 hours after injection.


19
18
5
4
3
2
1
^{125}I
T/S
4 C
1 — 17 C
Serum
Muscle
Liver
Bile
Spleen
Tail Kidney
HeadKidney
Gonad
Skin

consistent difference for all tissues between the mean T/S ratios of the two temperature groups. In all cases except skin and gonad, the T/S radioiodide values are greater for high temperature fish. In contrast, skin T/S values are much higher in the low temperature group. Samples of skin from the same fish were analyzed for stable iodide ^{127}I and showed the following values. The low temperature fish had a mean skin ^{127}I concentration of 5.21 µg/g with values ranging from 3.22 to 8.05 µg/g, whereas the mean for the high temperature group was 2.18 µg/g with a range from 1.83 to 2.68 µg/g.

Serum and skin samples were collected from burbot at all seasons at Lac Ste. Anne, Alberta and analyzed for stable iodide (^{127}I) to determine if a significant fluctuation of iodide occurred. The mean values are presented in Fig. 21. The numbers above the points represent the number of fish analyzed. The points were connected by a smooth curve to indicate trends. Part of the data was submitted to analysis of variance (plotted as ✱ in Fig. 21). The remaining samples were not included in the analysis because they contained insufficient numbers of animals to provide valid comparison. The analyses of serum ^{127}I values showed that there were significant differences between three seasonal samples.

$$F = 3.31 > F\ .025 = 2.90$$

The differences between sexes were not statistically significant. Skin values showed seasonal differences which were statistically significant.

$$F = 4.17 > F\ .05 = 2.86$$

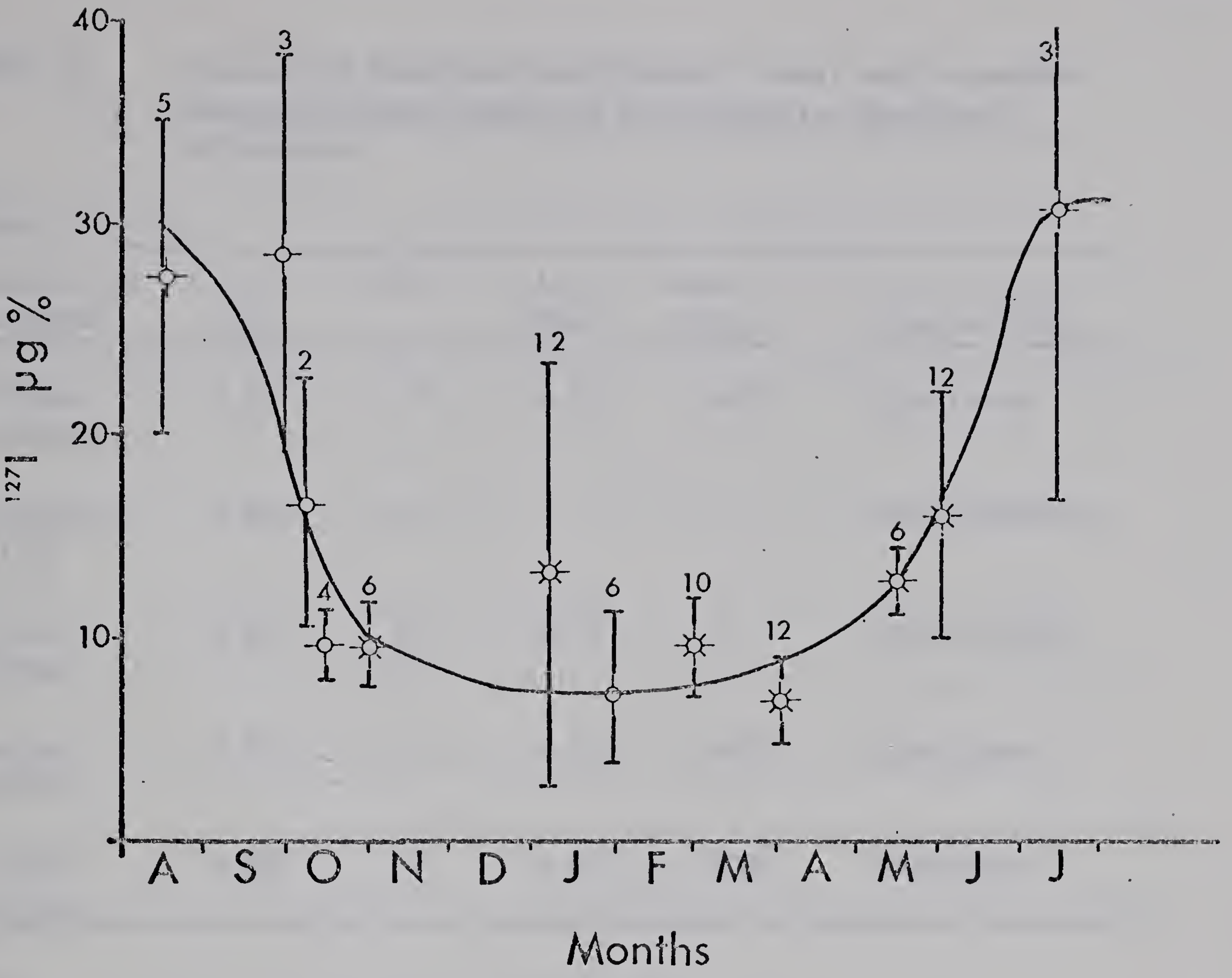
Differences between males and females were again not statistically significant.

Fig. 21A.

Seasonal changes in serum iodide ^{127}I concentration of burbot collected at Lac Ste. Anne, Alberta. Values used in analysis of variance plotted as ☼ Numbers indicate sample size. Bars equal one standard deviation.

Fig. 21B.

Seasonal changes in skin iodide ^{127}I concentration of burbot collected at Lac Ste. Anne, Alberta. Values ☼ were used in analysis of variance and numbers indicate sample size.


40
30
20
10
127I µg %
5
3
2
4
6
12
6
10
12
6
12
3
A S O N D J F M A M J J
Months

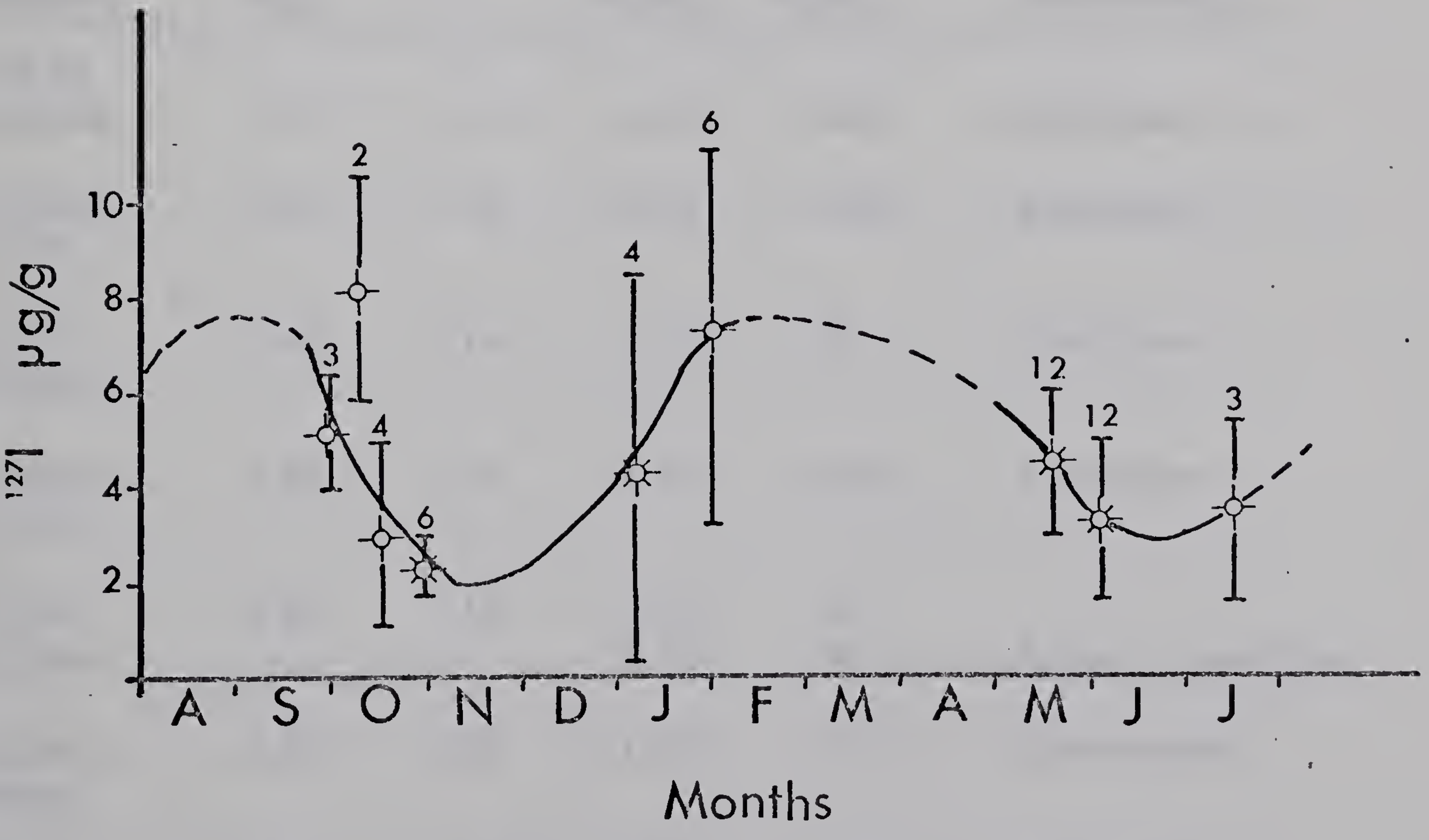

10
8
6
4
2
127I µg/g
2
3
4
6
4
6
12
12
3
A S O N D J F M A M J J
Months

Table 13. Summary of statistical tests (Student's t-test) used to compare means of seasonal serum and skin samples for significant differences.

Serum

Groups Compared	t calc.	d.f.	t tabled	level of sig.	Interpretation
17/8/66 29/10/66	5.54	9	4.78	.001	Significant
29/10/66 5/1/67	1.85	16	-	-	Not Significant
5/1/67 1/4/64	2.01	22	1.72	.1	Questionable
1/4/64 18/5/66	5.59	16	4.02	.001	Significant
18/5/66 15/7/66	4.49	7	4.03	.005	Significant

Skin

Groups Compared	t calc.	d.f.	t tabled	level of sig.	Interpretation
30/9/66 29/10/66	4.71	7	4.03	.005	Significant
29/10/66 5/1/67	3.55	18	3.20	.005	Significant
5/1/67 28/1/67	2.43	18	2.10	.05	Significant
29/10/66 28/1/67	2.82	10	2.63	.025	Significant
28/1/67 18/5/66	2.05	16	1.75 2.12	.10 .05	Probably Significant
18/5/66 2/6/66	1.87	22	1.72	.10	Questionable
28/1/67 2/6/66	2.87	16	2.47	.025	Significant
2/6/66 30/9/66	1.66	13	1.35	.20	Questionable

Student's t-test was used to determine if differences between specific sample means were significant. The results of these comparisons (Table 13) show that the 65% fall in serum iodide concentrations from 27.5 to 9.5 µg% between 13 August and 29 October, when the lake was cooling, is highly significant ($P \leq .001$). From October to April when the lake temperature was low (1 to 2 C) the serum iodide values remained fairly constant. As the lake warmed in the spring the serum iodide again increased until it reached a concentration which was more than double the April value ($P \leq .001$). By July the serum iodide concentrations had increased again ($P \leq .005$) to more than four times as great as the winter values. The remarkable resemblance of the seasonal serum iodide curve (Fig. 21A) to the temperature of the water in Lac Ste. Anne (Fig. 15) suggests that the changes are a direct response to environmental temperature.

The graph of skin iodide levels, unlike the serum iodide curve, shows a biphasic change. By 29 October the values had fallen to 45% of their September level ($P \leq .005$) showing the same pattern as was seen for serum values (Fig. 21A). However, by 28 January the skin iodide concentrations showed a three fold increase. ($P \leq .025$) to a level which is not significantly different from the autumn values. From 28 January to 2 June the skin iodide values again decreased ($P \leq .025$) to a level which is 45% of the midwinter high value. The obvious explanation for the decline in both serum and skin iodide levels in the autumn is the cooling of the water temperature in the lake. The subsequent increase in skin values could represent acclimation to lower temperatures but this leaves no interpretation for the second decline in skin iodide seen in the spring. The subject will be considered below.

The studies of Huang (1967) showed seasonal changes in the relative amounts of free inorganic iodide and protein bound inorganic iodide in the blood of fish. Because temperature is known to affect the equilibrium between bound and free ions (Klotz, 1950; Huang, 1967) T/S ratios for skin were calculated using the mean seasonal ^{127}I values; these are given in Table 14. During the period 28 January to 15 July T/S ratios for skin (based on ^{127}I values) show on inverse relationship with temperature (as temperature increases the skin T/S values decline). During the period October to January this relationship did not occur (Table 14). The complexity of the situation indicates that changes in a binding phenomena can explain only part of the observed changes in T/S ratios. Other factors, at present unknown, appear to be involved.

Table 14. Relationship between environmental temperature and T/S ratios for the skin of burbot from Lac Ste. Anne. Values are based on mean ^{127}I concentrations of seasonal samples.

Date	Temp. C	T/S (^{127}I)
30/9/66	13	.18
7/10/66	12	.50
14/10/66	8	.30
29/10/66	4	.24
5/1/67	1	.33
28/1/67	1	1.04
18/5/66	9	.34
2/6/66	12	.21
15/7/66	20	.11

DISCUSSION

CHARACTERIZATION OF THYROID PROTEINASE

The diffuse nature of the teleost thyroid gland imposes an important limitation in this study. Since the thyroid follicles are scattered loosely around the ventral aorta, the gland cannot be removed free of the ventral aorta and surrounding connective tissue. Consequently, there is a definite possibility that non-thyroidal cells contribute enzymes to the extract. However, the high proportion of thyroid tissue, plus the fact that much of the extrathyroidal component is non-cellular material, make it probable that most of the enzyme activity measured is of thyroidal origin.

The gradual decline in the reaction rate of the enzyme after the first minutes of digestion, as seen during the action of the proteinase upon the thyroglobulin (Fig. 1A), is a common feature of hydrolytic enzymes, especially proteinases (Dixon and Webb, 1964). The cause may be attributed to one or more of several factors: 1) The enzyme may undergo autolysis or inactivation with time. 2) The complexity of the protein substrate being hydrolyzed may be a factor; some bonds in thyroglobulin are more susceptible to hydrolysis than others, so that a greater porportion of more readily attacked bonds are split during the early period of the digestion. Bonds hydrolyzed later in the incubation period are those that cannot be split as readily (Kress et al., 1966). Therefore, the reaction rate declines. 3) The products of the reaction may be inhibitory (Walter and Frieden, 1963), or the products may have a limited solu-

bility in TCA. The susceptibility of fish proteinase to inactivation by relatively low temperatures indicates that, of these possibilities, temperature inactivation is the probable cause.

The observations discussed in the preceeding paragraph help to explain why the proteinase activity failed to show a direct linear relationship with enzyme concentration (Fig. 18 - curves B and C). The intense color of the reagent blank observed when thyroglobulin was used as substrate, caused by the presence of endogenous proteinase in the thyroglobulin preparations, suggests that product inhibition may have been important. Walter and Frieden (1963) have reviewed this subject and emphasize that product inhibition is not only more common than realized but also effective at lower product concentrations than is usually appreciated. Since thyroglobulin hydrolysis occurs normally in the phagosomes from which products diffuse freely into the circulation, the enzyme could conceivably be affected when exposed to higher product concentrations during in vitro studies. The presence of inhibitors in the enzyme preparation could be at fault (Dixon and Webb, 1964). However, experiments in which the enzyme preparation was dialyzed against distilled water to remove possible inhibitors failed to reveal any change in enzyme activity. At the concentration of enzymes used in the studies the stimulatory effect of dialysis would be low and might not be distinguishable. The tap water dialysis experiment suggests that either the removal of an inhibitor or the addition of an activator (metal ion) occurred. Only the second explanation is consistent with the results of distilled water dialysis studies which failed to stimulate enzyme activity.

The lower activity of the enzyme on thyroglobulin, compared to hemoglobin (Fig. 1A), is also a common observation. Many catheptic proteinases show greater activity on hemoglobin than on other substrates (Press, Porter, and Cebra, 1960; Bodwell and Pearson, 1964; Furlan, 1966). The anomalous relative insensitivity of thyroglobulin to the thyroid proteinase has been noted by McQuillan and Trikojus (1953). The failure, in the present study, to demonstrate significant activities with either beef or fish thyroglobulin as substrate indicates that interspecific differences in the structure of thyroglobulin are not the explanation for the low rate of digestion.

The occurrence of significant inactivation at 30 C was initially surprising, yet, according to Sizer (1943), this is not uncommon. Ushakov (1964) has summarized recent work on temperature stability and emphasizes that enzyme inactivation temperatures are associated with the temperature range encountered by the animal in nature. Kusakina's (1962) data on cholinesterases in fish provide an excellent example, because the species he studied inhabited different thermal zones in Lake Baikal. Those species inhabiting warm waters had a more heat stable enzyme than did those inhabiting the cold depths.

The thermal stability of the fish enzyme is obviously lower than that of beef spleen cathepsin D, which is inactivated after 40 min at 60 C (Press, et al., 1960). By comparison, a cathepsin D preparation from beef thyroids loses 75% of its activity after 20 min at this temperature (Balasubramaniam and Deiss, 1965) although chicken muscle cathepsin D is stable when heated to 60 C (Iodice, Leong, and Weinstock, 1966). These differences demonstrate that the use of inactivation

temperatures as a characteristic of cathepsin D is limited since large interspecific differences occur. In fish, where the inactivation temperature varies with the thermal history of the fish, the usefulness of this criteria is more restricted.

The three pH curves in Fig. 2 demonstrate the influence of temperature on pH optima. The pH 3.25 enzyme is apparently more temperature sensitive than the pH 2.5 form. With longer incubation periods the pH 2.5 activity, which could have a higher Q_{10} than the pH 3.25 enzyme, shows a proportionately greater increase in activity and masks the peak representing the pH 3.25 enzyme. A pH curve similar to the 90 and 135 min curves of Fig. 2A was demonstrated by Llundblad et al. (1960) and interpreted as representing two separate enzymes while the same effect went unnoticed in the work of Ekholm, Smeds, and Strandberg (1966).

The pH curve for enzyme prepared from the thyroids of winter fish (Fig.2B) does not show the pH 3.25 enzyme. Reference to Fig. 17B shows that the activity at this pH was reduced during the winter; moreover, this enzyme from winter fish was more susceptible to thermal inactivation than was the same form from summer fish. These factors explain the lack of a pH 3.25 peak. The pH curves determined on hemoglobin and thyroglobulin show differences in the number of pH optima. Hemoglobin curve Fig. 2A, shows three peaks while thyroglobulin curve, Fig. 3A, shows only two. The incubation period for thyroglobulin was relatively long (6 hr) so that if an enzyme with pH optima between pH 3 and pH 4 was present and had a low inactivation temperature, it too might be obscured, as happened to the pH 3.25 enzyme (hemoglobin substrate) in Fig. 2A. The thyroglobulin curves (Fig. 3A, Fig. 3B part B) might show three peaks if the duration of the incubation period was reduced.

The problem of enzyme specificity may be involved. Reinwein (1964) showed that there was a thyroglobulin specific enzyme present in human thyroid extracts. It is possible that similar specificities exist in fish and that one enzyme is inactive on thyroglobulin.

Although tempting, it is not valid to use the lower radioisotope pH curve of Fig. 3B as proof for the suggestion that the activity of pH 3.25 enzyme has been obscured by long incubation periods. Both isotope curves are based on very low activities and are not entirely reliable. They do confirm the pH 3 peak, seen in Fig. 3A, and suggest the presence of a second peak at about pH 4.0. The low activity is a further indication that the resistance of thyroglobulin to digestion is real and not an artifact of the Folin Phenol enzyme analysis method. The difference in the curves which used thyroglobulin as substrate may indicate interspecific differences. The more probable explanation is that the lack of sufficient reliable observations over short enough incubation periods has obscured subtle features of the enzyme's characteristics.

The differences in pH optima obtained with different substrates is consistent with the results of other proteinase preparations reported in the literature (McQuillan and Trikojus, 1953; Llundblad et al., 1960; Reinwein, 1964). These authors have all shown changes in the pH optima of thyroid proteinase when different substrates were used.

The lowest pH optimum found for hemoglobin (pH 2.5) is on the extreme acid side of the range of values reported in the literature. The evidence presented

in Table 6 on the temperature stability of the fish proteinase provides an explanation. The susceptibility to inactivation at low pH is significantly reduced at low temperatures. Thus the practice in this study of analyzing enzymes at 25 and 30 C has protected the enzyme from pH inactivation. The result is a lower pH optimum than would be evident at higher temperatures (Fig. 11). At 37 C, the temperature used by the other workers, fish enzyme would be inactivated at pH 2.5 and probably even at pH 3 or greater. The optima for fish proteinase would shift, therefore, to a significantly higher pH value at 37 C. Conversely, if mammalian preparations were analyzed at 25 C they would probably show a shift in optima to lower pH values.

The three enzymes demonstrated on hemoglobin substrate are probably, though not certainly, of thyroidal origin. Multiple proteinases have been demonstrated at pH 3.75, pH 6.5, and pH 8.5 (Llundblad et al., 1960). The pH 8.5 activity is unusual; Pastan and Almqvist (1966a) and Jablonski and McQuillan (1967) are the only workers reporting such an occurrence. Pastan and Almqvist (1966b) have identified the mast cells in the rat thyroid as the origin of the pH 8.5 enzyme but the similar enzyme described for the pig (Jablonski and McQuillan, 1967) may not have the same origin. Other examples of multiple thyroid proteinases with different acid pH optima have been given by Alpers et al. (1955), Haddad and Rall (1960), and Shapland (1964), while Llundblad et al. (1960), Reinwein (1964), and Ekholm et al. (1966) have shown the presence of two forms with very similar pH optima.

Weiss (1953) described the presence of a metal dependent peptidase in beef thyroids. This was confirmed by McQuillan et al. (1954) whose work eventually led to the description (by Laver and Trikojus, 1956) of several peptidases from hog thyroids. Other authors have meanwhile demonstrated multiple proteinases (Llundblad, et al., 1960) and some lysosomal acid hydrolases (Ekholm et al., 1966; Jablonski and McQuillan, 1967). An overwhelming body of evidence has accumulated to show that many kinds of intracellular proteolytic enzymes are found in thyroid tissue. There is even some evidence to show that some of these exist in multiple forms.

Several studies have shown that purified cathepsin D and E are probably not activated by cysteine (Press et al., 1960; Lapresle and Webb, 1962). Iodice et al. (1966) have demonstrated the synergistic effect between cathepsin A and cathepsin D in muscle. The presence of several peptidases in mammalian thyroid preparations makes a synergistic relation between them and the proteinases likely.

The inhibition of fish thyroid extracts by EDTA and the stimulation after tap water dialysis suggest the presence of a metal dependent peptidase (Tables 2 and 3). Although the tap water might have removed an inhibitor further studies with distilled water failed to confirm this. The variation in the magnitude of the EDTA effect in different experiments reported in Tables 2 and 3 may be the result of the different procedures used or an indication of seasonal differences in the enzyme. Evidence for other seasonal changes in the enzyme is given in Fig. 17B and Table 12. It may be significant that

EDTA had its greatest effect on an enzyme preparation made from thyroids of summer fish (Table 2) while enzyme prepared from winter fish thyroids appeared more stable. Seasonal variation in the relative enzyme concentration and response to iodoacetate are seen in Fig. 17B and Table 12.

The apparent demonstration of a sulfhydryl dependent proteinase (Table 3) may be misleading. Lapresle and Webb (1962) and Press et al. (1960) have shown that neither of the two major intracellular proteinases, cathepsin D or E, appear to be sulfhydryl dependent. Purified thyroid proteinases have been unaffected by sulfhydryl reagents (McQuillan and Trikojus, 1953; Balasubramaniam and Deiss, 1965; Kress et al., 1966). Therefore, it appears likely that the stimulation of the burbot proteinase by sulfhydryl reagents is actually a response of a sulfhydryl dependent peptidase which is acting synergistically with a proteinase.

The evidence presented above suggests that a metal dependent enzyme and a sulfhydryl dependent enzyme may occur in fish thyroid extracts. It is not known whether these are peptidases or true proteinases. As a working hypothesis it is suggested that the composite pH curve (Fig. 2A) represents the total effect of the synergistic action of at least one proteinase and one or more peptidases. Because thyroid proteinases generally exhibit lower pH optima than thyroid peptidases it was assumed also that the pH 2.5 enzyme probably represents the optimum pH of the proteinase (Shapland, 1964; Jablonski and McQuillan, 1967). In addition it was assumed that, since the thyroid proteinase of the steer was of the cathepsin D type, the fish enzyme might be

a cathepsin D.

There is evidence to suggest that multiple forms of enzyme 1, 2, and 3 occur in the burbot. This evidence comes from the temperature inactivation studies (Table 7), the rate-temperature curves (Fig. 13), and the K_m determinations (Fig. 12). These all show seasonal differences in enzyme 1. Such differences are most simply explained by assuming that different forms of each enzyme are present at different times of the year.

The relative concentrations of hemoglobin splitting proteinases (cathepsin D) in different tissues have been reported by Kamner, Peranio, and Bruger (1950), Clements (1957), and Bouma and Gruber (1964). In the rat, levels of cathepsin D are found to decrease in the sequence: spleen > kidney > liver. The levels found in muscle and blood are much lower (Bouma and Gruber, 1964). Essentially the same pattern was found in the dog (Kamner et al., 1950), dogfish shark (Clements, 1957), and the burbot of the present study. The thyroids of the dog and the dogfish shark showed higher levels of enzyme than the spleen. The lower concentrations of enzyme in the burbot thyroid appears to be related to the nature of the thyroid. In the dogfish shark, as in the mammal, the thyroid is compact and encapsulated while in the teleost it is diffuse. Assuming that the burbot "thyroid sample" contains as much as 50% of the actual thyroid tissue, enzyme values per gram of thyroid tissue are of the same relative magnitude as found in the thyroids of the other species.

BIOLOGICAL ROLE OF PROTEINASE

It was not always possible to stimulate the fish thyroid with TSH because of the unresponsiveness of fish to mammalian hormones (Olivereau, 1954); (Fontaine and Fontaine, 1957). A lack of knowledge of the dose-response relationships of fish to TSH was probably involved (Powell, Rahman, and Deiss, 1964). Variability resulting from the diffuse nature of the fish thyroid and the seasonal variation of the physiological processes in the fish made the interpretation of the obtained results difficult.

Animals treated with TSH (Fig. 5A) showed a maximum stimulation of the thyroid proteinase at six hours. This compares favorably with the work of Deiss et al (1966) who showed that maximal incorporation of isotopically labelled thyroglobulin into colloid droplets occurred at six hours. Earlier, Poffenbarger et al. (1963) and Powell et al (1964) showed that the TSH stimulated release of radioiodide from dog thyroids was maximal from two to five hours after TSH injection. Taurog and Thio (1966) found that two hours were required for maximal release of iodide from the rabbit thyroid. The reason for gradual increase in activity of the saline injected control burbot thyroids (Fig. 5A) is not evident. The effect may have physiological significance since it can be seen in some experiments done by Deiss et al. (1966). The simplest explanation is that it might be the result of stress induced by handling, which has been shown to have complex effects on the thyroid of mammals (Ducommun et al., 1967).

At low temperture (5 C, Fig. 5B) TSH had no effect on thyroid proteinase activity. This may have been caused by the relative ineffectiveness of mammalian TSH at low temperatures (Olivereau, 1954). However, the positive results with fish pituitary extracts support the observations of Fontaine and Fontaine (1957) that fish TSH is equally effective on fish at low or high temperatures. An alternative explanation is that the fish extract had a much higher potency than the mammalian TSH. The rapidity of the response to fish pituitary preparations indicates that fish are capable of responding as quickly as mammals to TSH. It is interesting to note that the increase in enzyme activity seen in the controls of Fig. 5A did not occur in the study shown in Fig. 5B.

Fish pituitary extract failed to stimulate thyroid proteinase activity in April, 1967 (Fig. 6), suggesting low TSH potency in the extract. These fish, unlike the animals used in the study of Fig. 5B, also failed to show the expected thromboplastic response. Freezing may have inactivated the hormone. In June, 1966 a previously frozen preparation of mammalian TSH failed to stimulate thyroid proteinase when other fish of the group seemed particularly sensitive to mammalian TSH.

DeRobertis (1941) first studied the thyroid proteinase in the rat , using a semiquantitative method. He showed that the enzyme activity varied with the functional state of the gland and that it responded to exogenous TSH. Dziemian (1943) confirmed this work with quantitative methods as did Watson and Trikojus (1957). Loughlin, McQuillan, and Trikojus (1960) extended the

work to show that TSH also increased the levels of a thyroid peptidase. With this background it was concluded that the thyroid proteinase mechanism occurs in fish as well as in mammals.

Mammalian studies have not always shown stimulation of thyroid cathepsin after TSH. Irie (1962), for example, could not demonstrate stimulation even when TSH was administered to the living animal. His use of a non-optimal pH probably reduced the activity levels while his use of propylthiouracil may have inhibited the enzyme. The use of MMI in the present study (Table 3) inhibited the enzyme, as did urea in the 1954 study of McQuillan et al.

The mechanism by which TSH stimulates proteolysis in the thyroid is unknown. It is possible that the hormone acts directly on the enzyme. However, failure to demonstrate proteinase stimulation after TSH treatment is a common result of *in vitro* studies (Alpers and Rall, 1957; Litonjua, 1960; Irie, 1962). The *in vitro* treatment of burbot thyroid proteinase with TSH gave negative results (Fig. 10, Table 5). In fact, the TSH actually inhibited the proteinase. Alpers and Rall (1957) had earlier shown a similar effect on mammalian proteinase with one specific TSH preparation. The results of a number of studies in which animals were treated with pituitary preparations have been discussed by Pickford and Atz (1957). Since these authors note that inhibitory substances are found in some pituitary preparations it seems reasonable to conclude that some component found in the TSH preparations used in the present study acts as an inhibitor. The results discussed above contrast with the studies of Reinwein (1964) who appears to have

shown increased enzyme activity when the TSH was added to the thyroid extract. Reinwein has emphasized that TSH stimulation occurred only when thyroglobulin was used as a substrate.

The mechanism of TSH activation has yet to be explained. The results of Poffenbarger et al. (1959) who showed increased proteolysis in surviving thyroid slices after prior *in vivo* or *in vitro* TSH treatment suggests that some cellular organization is required. The work of Wollman et al. (1964) showed that one of the early effects of TSH must be the stimulation of the cell membrane to initiate pinocytosis. Simultaneously, the transfer of lysosomal enzymes to the pinocytotic vacuoles is stimulated. The attempt to demonstrate this in the burbot (Fig. 7, 8, and 9), although not conclusive, indicated that lysosomal enzymes may be transferred to more fragile phagosomes. The results of the fish study showed an increase in the total enzyme activity after the thyroid had been stimulated by the injection of TSH into the fish. Taurog and Thio (1966) and Schneider and Goldberg (1965) have shown that the increase in enzyme activity in response to TSH (in the mammal) does not depend upon protein synthesis; the increase in burbot therefore appears to have resulted through the activation of pre-existing enzyme.

The indication that TSH stimulation may increase the activity of the pH 3.25 enzymes proportionately more than the pH 2.5 enzymes is supported by the work of Deiss et al. (1966). These workers have shown recently that the pH optimum for thyroglobulin proteolysis in phagosomes is increased by TSH stimulation.

Similar findings by DeRobertis (1941) have been overlooked in the literature. According to Deiss et al. (1966) the effect does not occur if the enzyme is free in solution. This implies that the integrity of the phagosome particle is an important factor and that pH optima determined for enzymes in solution may bear little relation to the pH optimum under physiological conditions. Nevertheless, the estimation of total or free lysosomal enzyme appears to be a valid index of thyroid activity.

DIFFERENCES IN PROTEINASE FROM WARM AND COLD ACCLIMATED FISH

Fig. 11 shows that the optimal pH for hemoglobin digestion for the burbot thyroid is temperature-dependent. The optima of both enzyme 1 and enzyme 3 shift towards lower pH values as temperature is reduced. Fig. 11 (B and C) suggest that enzyme 2 is present in both winter and summer extracts and that it shows the same shift in pH optima with decreasing analysis temperature. Because of the methods used in preparing extracts and in presenting results no significance can be attached to the quantitative differences in enzyme activity. The observed change in pH optima is probably related to differences in stability of the enzyme. At low temperatures (0 C) the enzyme is relatively stable at very acid pH values (pH 2.0) but at high temperatures (30 C) the enzyme is somewhat inactived even at pH 7 (Table 5). Press et al. (1960) have suggested a similar explanation for the inactivation of mammalian cathepsin D below pH 3.

Fig. 11 shows that there are no significant differences in the pH optima of enzymes extracted from winter and summer fish. This is best seen in Fig. 11

part B (pH 2.0, enzyme 1). A remarkable feature of the data is the extremely acid pH optima at low temperatures. The work of McQuillan et al (1961) and Stoll et al. (1959) have shown that the synergistic action of other enzymes with the proteinase results in a significant release of iodoamino acids at pH values above the optimum of the proteinase. In some cases the effect of the other enzymes (peptidase, α amylase, glucuronidase) is to maintain the hydrolysis rate at a constant value even though the digestion may occur several pH units above the optimum of the proteinase. The extremely acid "non physiological" pH optimum of the proteinase has been considered a major problem by several workers in spite of the demonstration by Rous (1925) that the pH in digestive vacuoles of monocytes, as in protozoan food vacuoles (Prosser and Brown, 1961) is very low (pH 1 to 4). The current realization that a similar situation may occur in thyroid cells (Wollman et al., 1964) should help to correct the earlier misconception.

The sensitivity of the thyroid proteinase to temperature inactivation suggested that inactivation studies might provide useful information about seasonal changes in enzyme. Enzyme prepared from winter fish was much more sensitive to heat than enzyme extracted from summer fish (Table 7). Furthermore, the pH 3.25 enzyme was more heat sensitive than the other two. Thus the differences in winter and summer pH curves (Fig. 2) determined at 30C, are directly atttributable to differences in temperature stability. The increased stability in summer may have functional value in promoting survival at extreme temperatures. It is possible that the differences are simply a concomittant effect of change in some other function of the enzyme such as its activation energy.

The rate-temperature curves for the thyroid proteinase of the burbot (Fig. 13A and B) are unlike most rate-temperature curves seen in the literature. Contrary to the general rule (Sizer, 1943) fish proteinase curves show neither high Q_{10} values at low temperatures nor decreasing Q_{10} values at high temperatures. They, therefore, contrast sharply with the curves for whole animal or tissue respiration shown by Freeman (1950) and Peiss and Field (1950). Rieck, Belli, and Blaskovics (1960) have shown similar curves for the total oxygen consumption for frogs, Rana sp. Unfortunately, they offered no explanation for the observed effect. The constancy of Q_{10} values at higher temperatures for burbot enzyme is similar to the results of Peiss and Field (1950) on the Arctic cod (Boreogadus). The restriction of the analyses (Fig. 13) to relatively low temperatures may be a partial explanation for the failure to demonstrate decreasing Q_{10} values at high temperatures.

The burbot thyroid proteinase curves resemble curves obtained by Massey (1953) for the action of fumarase at different temperatures in alkaline solutions. Dixon and Webb (1964) have noted that, to their knowledge, Massey's curves were unique in that the slope increased above the critical temperature. There are several mechanisms which could produce the biphasic rate-temperature curve. The simplest explanation is that there are two or more enzymes present with different Q_{10} values. The presence of more than one type of enzyme in burbot extracts has been demonstrated in Fig. 2A and Table 2. Seasonal changes in the nature of the enzymes (Fig. 12, 13, 17 and Table 7) suggest that there is probably more than one form of each enzyme. The possible existance of isozymes

arises. Multiple forms of cathepsin D have been demonstrated by Press et al. (1960). Proteinase isozymes could explain not only the biphasic rate curve (Fig. 13) but also the seasonal differences observed in Fig. 12, 13, 17 and Table 7. Unfortunately, there seems to be no conclusive demonstration of proteinase isozymes.

On the other hand, a single enzyme could be involved. Massey (1953) has argued that a temperature dependent dissociation of an enzyme into active subunits possessing different activation energies than the parent molecule would produce a biphasic effect. Several proteinases have been shown to have structural subunits. (Reithel, 1963; Klotz, 1967). It is apparently not known whether the subunits retain functional activity. Sizer (1943) outlined other mechanisms by which temperature could induce changes in an enzyme. The altered response of the enzyme to a variety of factors such as temperature, pH, ionic strength, and specific ions could produce biphasic curves. This aspect of the present study requires and deserves more study.

A remarkable feature of the rate-temperature curves is the occurrence of low Q_{10} values at low temperatures. The extracts on which these studies are based were made from the thyroids of fish collected between January and July. During most of this time the lake temperature was generally cold, gradually warming to about 5C at the end of April. From 1st May to mid July temperature increased rapidly to about 20C. Precht (1958) feels that the development of enzymes with low Q_{10} values is an important acclimation mechanism in that the effect of temperature variations on metabolic processes

is reduced. Thus enzymes with relatively low Q_{10} values could help to stablize metabolic processes in the burbot during a period of temperature change.

The biphasic pattern of curves 2, 3, and 4 (Fig. 13 A and B) which indicate a seasonal change in the critical temperature suggests that curve 1 might have shown a similar effect if the analyses were carried out at lower temperatures. Assuming this to be true, the critical temperature in January would be at 5 C or lower. During the period January to July the critical temperature increased parallelling the increase in the lake temperature. At temperatures below this point the proteinase is relatively unaffected by small variations in temperature. Thus the development of enzyme with a higher critical temperature represents a means of stabilizing proteinase activity at higher temperatures. Therefore, the changes seen in Fig. 13 can be interpreted as indicating acclimation to warmer temperatures. The initiation of these changes in January and February is puzzling. Ushakov (1964), in his review on the effects of temperature on proteins, noted that the thermal stabilities of the proteins and enzymes of poikilotherms frequently change during periods of reproduction or at times of hormonal change . In the years 1965 to 1967 burbot at Lac Ste. Anne spawned during the middle of March, so that the change in the temperature characteristics of the thyroid proteinase are correlated with the reproductive period. Considering the year to year variations in the date at which Lac Ste. Anne begins to warm and the rapidity of the process once the ice is gone, the initiation of acclimation to warmer temperatures in February is reasonable.

If the changes in critical temperature are related to the process of temperature acclimation it would be mid June (under natural conditions) before the enzyme would operate above its ciritical temperature. If the critical temperature indicates the limit of acclimation the failure of July enzyme to show a higher critical temperature than 15 C may indicate that 15 C is the upper limit for capacity acclimiation in the adult burbot. That is, the animal cannot acclimate so as to maintain usual levels of metabolism at temperatures higher than 15 C. Observations at Lac Ste. Anne have indicated that very few burbot are caught after the end of June. Since the trap net depends upon the movement of the fish it appears that burbot are less active during the summer, as has been reported by Nikolsky (1963).

Although the burbot appears to have a limited capacity to acclimate to high temperatures, its ability to function at low temperatures is outstanding. The burbot, being of Arctic origin (Zenkevitch, 1963), is probably basically adapted for cold conditions. The performance of reproductive activities at temperatures of O C to 3 C plus the observation that the optimal temperature for the development of the young is O C to 3 C supports this (Evropeiziva, 1947), as do the observations that the burbot retires to the cold depths of lakes during the summer (Hubbs and Lagler, 1947; Lawler, 1963). Evidence consistent with this interpretation is obtained from Fig. 13B. These curves show that the activation energy of the enzyme is lower at low temperatures, suggesting a more efficient operation under these conditions. The significance of the slope of the rate-temperature curve above the critical temperature is not clear. In nature, animals would

not operate at temperatures of this range until June. During most of the year (October to June) the special properties of the enzyme exhibited at these temperatures would be non-functional. This does not preclude the possibility that the thyroid proteinase is important in high temperature resistance acclimation.

IODIDE DISTRIBUTION STUDIES

Hickman (1962) has evaluated the role of environmental factors in iodide metabolism. His work clearly demonstrates that in fish, there may be large seasonal variations in environmental and body iodide levels. The mean concentration of iodide in Lac Ste. Anne is 0.154 µg/liter, much lower than the concentration fround in the ocean (43 µg/liter). The recent Pleistocene (Zenkevitch, 1963) origin of the burbot from a marine form in an iodine rich habitat made a knowledge of the burbot's iodide metabolism a necessity. If the animal was exposed to chronic or seasonal dificiencies in iodide variations in the thyroid activity (Hickman, 1962; Eales, 1963) could result.

The initial T/S ratio in Fig. 18 and 19 seem to be related, in part, to the blood supply of the various tissues. T/S values of kidney and gill (Fig. 19), which are well supplied with blood, were fairly high initially and showed little change with time. Radioiodide penetrated the brain more slowly; tissue levels were never as high as kidney. This may indicate that iodide is largely excluded from the cerebral spinal fluid (Wallace and Brodie, 1939). The pattern for muscle was different. The increased T/S ratios in muscle after three days may indicate that labelled thyroid hormone had begun to enter the

the circulation. Tata (1964) has shown that mammalian muscle, although having a low thyroxine turnover rate, may accumulate significant amounts of hormone.

The burbot's skin rapidly accumulated extremely high concentrations of radioiodide (Fig. 18). Peak values occurred at 30 hr and had declined significantly by 72 hr. Gennaro and Clements (1956) found that iodide was concentrated by the dark dorsal skin of the frog to a greater extent than by the paler ventral skin and that the iodide was incorporated into moniodotyrosine (MIT) diiodotyrosine (DIT). Taurog and Evans (1967) have recently confirmed the existence of an extrathyroidal synthesis of thyroxine in the rat. Their work suggests that the iodide accumulated in the skin of the burbot could be iodinating tyrosine which might be released to the circulation. McNabb (1963) found evidence to suggest that the skin of the rainbow trout was capable of synthesizing thyroxine. However, in view of the report of Jacoby and Hickman (1966), who showed that iodophenols underwent a spontaneous iodination with radioisotope (exchange labelling) on chromatograms under the conditions used by McNabb, the validity of McNabb's findings is uncertain.

Other factors are involved in the secondary decline in skin T/S ratios. Probably a redistribution of radioiodide among tissues occurs. The decline in T/S ratios will be accentuated by the use of total serum radioiodide concentrations (instead of the actual inorganic iodide values) in the calculations. The apparent inorganic concentration (approximated by the total radioiodide in the serum) will be greater than the true value because small amounts of radiohormone will be present. During the early part of the study this effect will be

negligible; later, when the fraction of radiohormone begins to increase, the T/S ratio will appear to decrease.

The results of stable iodide analyses of serum and skin samples collected at all seasons from Lac Ste, Anne burbot and presented in Fig. 21 show major changes during the course of the year. The stable iodide concentration in burbot serum yielded values ranging from 7 µg% to 30 µg%. By comparison, values for skin ranged from 227 µg% to 816 µg%. The values for skin are remarkably high, equaling or exceeding those reported by Vinogradov (1953) for 12 species from both marine and fresh water habitats. Members of the order Gadiformes were the only teleosts having skin iodide concentrations similar to the burbot; only the marine cod (Gadus morrhua and Gadus aeglefinus) had higher values than the burbot. The ability of the burbot to maintain, in the skin, concentrations of iodide many times the concentration of sea water is remarkable. These concentrations are even more remarkable when it is realized that the iodine content of Lac Ste. Anne is 0.3 to 0.4% of that in the ocean (Hickman, 1962). Vinogradov (1953) has noted that quite different values have been obtained for the iodide content of cod skin by American and European workers. He suggests that the food of the two populations may have a different iodide content. Differences in food supply may be important but in an environment as rich in iodide as the sea it would not appear that iodide values were limited by the availability of iodide. In view of the burbot's seasonal changes in iodide it seems likely that seasonal changes related to some aspect of the cod's physiology may be the cause of the differences.

Of the other fish species from Lac Ste. Anne which have been studied, only the whitefish (Coregonus clupeaformis) has higher blood values (16 - 48 µg% - Hickman, 1962). Lower values were found in the sucker, Catostomus commersonii (2 µg%), the pike, Esox lucius (11 µg%), and the walleye, Stizostedion vitreum (5 - 9 µg% - Hickman, 1962, 1967). The high blood iodide concentrations in the whitefish are caused by the presence of a plasma protein fraction which binds inorganic iodide (Huange, 1967). The bound iodide may serve as a storage depot for inorganic iodide (Leloup and Fontaine, 1960), a function which would especially advantageous if a fish inhabited a low iodide environment or experienced significant seasonal differences in iodide requirements. Although burbot blood does not bind inorganic iodide (Huang, 1967) its skin may serve as a reservoir of iodide.

One aspect of the work of interest is the demonstration that significant amounts of iodide are lost from the serum and skin during the fall. If these losses are severe enough the animal could experience a deficiency of iodide which might produce a compensatory increase in thyroid activity. Estimates of the amount of iodide lost are presented in Table 15. These values were calculated by assuming that the blood volume of the burbot equals that of the cod, Gadus morrhua (Ronald et al., 1964). Skin was assumed to represent 10% of the body weight. The Table shows that a loss of 600 µg + 5.3 µg or 605.3 µg of iodide would occur from a 1 Kg burbot during a two month period in the autumn.

A loss of iodide at the same time as a rapid change in environmental temperature could be associated with a disturbance in ionic regulation. Hick-

Table 15. Results of calculations made to estimate the amount of iodide lost from skin and blood over a period of 2 months in the fall.

organ	wt. in 1 kg fish gm	change in iodide conc. initial µg%	final µg%	change µg%	total iodide µg
skin	100	800	200	-600	600
blood	24	30	8	-22	5.3
					-605.3

man (1962) suggested that excretion of iodide might exceed uptake at low temperatures. Woodhead and Woodhead (1959) showed that cod exposed to 2 C water developed an ionic imbalance that was often lethal. Wikgren (1953) noted that the effect of low temperature on ionic balance appeared to be caused by an inhibition of the active uptake mechanism by cold. Thus a failure to replace ions as rapidly as they are lost through diffusion could explain the disappearance of iodide from the burbot. This loss would be accelerated if dietary intake of iodide was also reduced at low temperatures (Barrington, 1957). However, since burbot digestion is supposed to be optimal at 10 C (Gomazkov, 1959), and burbot caught at Lac Ste. Anne in the fall and early winter were usually gorged with food, nutritional effects are probably not a significant factor.

The acclimation studies (Fig. 20 and Table 15) showed an inverse relationship between T/S ratios for skin and temperature, suggesting that seasonal changes in serum iodide might be associated with changes in the equilibrium between skin and serum (Klotz, 1950). The relationship is demonstrated by the data presented below. On 22/7/66 fish were transported from Lac Ste. Anne and held overnight at temperatures 10 to 12 C below lake temperature (18 C). When analyzed, serum iodide concentrations had decreased 42% and skin iodide increased to 120%, relative to samples collected at lake temperatures a week earlier. Probably an equilibrium exists between skin and blood iodide and the binding of iodide is increased at low temperatures. Klotz (1950) showed that this is possible but the magnitude of the temperature effect demonstrated in the burbot is much greater than those shown by Klotz (1950) or Huang (1967). A simple

change in an equilibrium cannot explain completely the complex biphasic changes in skin iodide (Fig. 21B).

Calculations were made to evaluate the possibility that the skin iodide might be utilized by the thyroid. It was assumed that 23% of the iodide entering the thyroid gland would be synthesized into iodothyronines. If all the skin iodide lost over a two month period was accumulated by the thyroid 140 µg of iodide or 222 µg of iodothyronine would have been produced. If all this was secreted as hormone the secretion rate would be 0.37 µg/day/100 g. This value compares favorably with rates given by Leloup and Fontaine (1960), who reported values of 0.18 µg/day/100 g for resting rainbow trout and 0.35 µg/day/100 g for active fish. Thus the depletion of skin iodide in the autumn could indicate an increased synthesis of thyroid hormone, a conclusion which is supported by the enzyme data in Fig. 17. The recovery of skin iodide levels in January would indicate that the thyroid was no longer drawing upon the iodide reserves.

SEASONAL CHANGES IN PROTEINASE ACTIVITY IN RELATION TO TEMPERATURE ACCLIMATION

The interpretation of the significance of the seasonal changes in thyroid proteinase activity seen in Fig. 17A and 17B must account for:

low levels of thyroid enzyme activity during the summer and winter.

a relatively high activity during the fall.

a transitory increase in the spring.

It is apparent that the periods during which the enzyme activity is high are also

the times when the temperature of the aquatic environment is changing. This supports Hickman's (1959) suggestion that the thyroid might be important during periods of environmental stress.

There seem to be two possible interpretations for the data. Increased amounts of thyroid hormone may be required only during the process of acclimation. It is possible that once acclimated burbot would require only low levels of thyroid hormone. The transitory nature of the increased thyroid activity in Fig. 17A supports this view. The data of Fig. 6 suggested that TSH stimulation is probably greater during the fall. The work of Hoffert and Fromm(1959) on rainbow trout previously discussed, showed a relative increase in the thyroid hormone secretion rate at low temperatures. This increase was measured before the trout were completely acclimated. Therefore, it is possible that the increase in thyroid enzyme in the fall serves to initiate the acclimation process. It seems that increased levels of thyroid enzymes maintained during the fall result from increased TSH secretion. After the animal has acclimated TSH production decreases and thyroid enzyme activity falls.

A similar explanation may apply to the increased activity of thyroid proteinases seen in June (Fig. 17). If thyroid hormone is important in the process of acclimation an increase in hormone production (and an increase in proteinase activity) could be expected in both spring and fall. The depletion of skin iodide stores in the spring could be another indication of increased thyroid activity at these times. The reason for the short duration of the peak thyroid enzyme activity in spring may be related to the differences in the process of acclimation to low

temperature as opposed to acclimation to high temperature. Konishi and Hickman (1964) presented evidence to support the idea that acclimation of neural processes to cold and heat by rainbow trout may not involve the same mechanisms. In fact, some aspects of neural function may not acclimate to higher temperatures.

Another possible explanation is that the June response could represent an overshoot of thyroid metabolism in response to increased temperatures. This could reasonably be expected since the temperature in June would reach 10 to 15 C. Fig. 13 has demonstrated that temperature variation during the summer (when the temperature is above 10 to 15 C) will have a relatively greater effect on the activity of the enzyme than the same temperature change occuring below the critical temperature. That is, at temperatures exceeding 10 to 15 C the enzyme has a higher range of Q_{10} values. Since temperature dependent overshoots in fish metabolic processes may last for weeks (Grainger, 1958) the apparently extended duration of the response by burbot enzyme poses no problem to this interpretation.

The preceeding interpretation has a major weakness. It fails to take into consideration the differences between enzymes from summer and winter fish. This study has clearly demonstrated that differences in thermal stability, temperature coefficient, Michaelis Constants, and cofactor relationships exist. These force one to conclude that different enzymes are involved. In addition to these differences, Fig. 17B shows that the relative concentrations of the different thyroidal enzymes change. Although the changes in enzyme might

represent enzyme acclimation for efficient functioning at low temperatures there is another possibility, that is, low temperature acclimation of the thyroid release system might result in a change in the nature of the thyroid hormone released which in turn would alter the nature or relative concentration of circulating thyroid hormones.

There is evidence in the literature to indicate that changes in the composition of the circulating thyroid hormone does in fact, occur. Laver and Trikojus (1956) showed that a carboxypeptidase from porcine thyroids preferentially released diiodtyrosine, whereas the proteinase preferentially released monoiodotyrosine. It is probable that the release of triiodothyronine and thyroxine would show similar differences. Albright, Heninger, and Larson (1965) showed that cold induced hyperthyroidism caused a change in the relative amounts of circulating triiodothyronine (T3) and thyroxine (T4). In the rat the T3/T 4 ratio increased. Gorbman (1959) and Berg, Gorbman, and Kobayashi (1959) have shown that in Umbra sp. the production of labelled thyroxine was very high at low temperatures. Moreover, another series of studies showed that the synthesis of triiodothyronine relative to thyroxine was increased at low temperatures. These results, although complicated by differences in season, species, and iodide availability (Leloup and Lachiver, 1955) demonstrate that, in fish, as in mammals the relative T3:T4 concentration is important. Some idea of the significance of the increase in T3 concentration can be obtained from Hochachka's (1962) work. This demonstrated not only that thyroid hormone and low temperature acclimation have the same effect on the pentose phosphate pathway but showed also that T3 was much more effective than thyroxine.

Therefore, one can conclude that temperature acclimation of the thyroid may occur in two ways. During periods of rapid temperature change increased production of TSH by the pituitary could maintain thyroid activity at the required level until acclimation at the cellular and enzyme levels occurred. These changes, would result in an alteration of the ratio of the two thyroid hormones. As a result a more effective form of hormone, rather than an increased concentration of hormone,would satisfy the needs of the organism.

SUMMARY

1. Extracts of the thyroid tissue of the burbot, *Lota lota* L. contain at least three proteolytic enzymes with different acid pH optima, as measured on hemoglobin substrate. These are called enzyme 1, 2, and 3 with enzyme 1 being that with the most acid pH optima.
2. The activity of the enzymes was studied using bovine hemoglobin, bovine thyroglobulin, and fish thyroglobulin as substrates. The activity of the enzyme is much greater on hemoglobin.
3. The optimum pH values vary with temperature. Generally three optima are shown for hemoglobin hydrolysis while only two are evident when the enzyme acts upon thyroglobulin. The optima for thyroglobulin substrate tend to be at higher pH values.
4. The concentration of enzyme 1 in different tissues has been shown to increase in the sequence blood < muscle < thyroid < liver < kidney < spleen.
5. During temperature acclimation the concentration of enzyme 1 in most tissues remains unchanged. Spleen enzyme shows a significant increase in the low temperature group while liver proteinase from the high temperature group shows higher activities.
6. The enzymes are significantly inactivated (27%) by exposure to 30 C for one hour at neutral pH. Although the enzyme is susceptible to acid denaturation the effect is reduced at lower temperatures.
7. The pH optima for the three enzymes (hemoglobin) are not constant. These are higher at 30 C and decline with decreasing temperature.

8. The proteinases are stimulated by cysteine and inhibited by iodoacetate, glutathione, ferrous ions, MMI, and EDTA at concentrations of 0.01 M.

9. Seasonal differences in the extent of iodoacetate inhibition of enzyme 1 have been demonstrated.

10. The injection of TSH into burbot produces an increase in proteinase activity within a few hours. Maximal stimulation occurs at about 6 hours at 15 C.

11. Doses of mammalian TSH effective at 15 C fail to stimulate the fish thyroid at 5 C. At this temperature crude fish pituitary extracts were effective.

12. Purified TSH acted as a proteinase inhibitor when added to enzyme systems *in vitro*.

13. Studies on enzymes from fish collected in summer and winter showed that the enzymes were not the same although they possessed the same pH optima.

14. Enzymes from fish caught in the summer showed less inactivation (5 - 6%) at 37 C than did enzymes from fish caught during the winter which showed a loss of 45% of the initial activity.

15. The Michaelis constant for enzyme 1 from fish caught in summer is 0.154 g%, higher than the K_m for the same enzyme extracted from winter fish, which had a K_m of 0.137 g%. The determination was done on hemoglobin substrate (pH 2.5) at 30 C.

16. Changes in the rate-temperature relationships of the enzymes occurred. The curves showed a discontinuity with higher temperature coefficents above the

critical temperature. During the period January to July the critical temperature increased from below 5 C to 15 C.

17. Changes in radioiodide T/S ratios for various tissues at intervals after injection and at different temperatures were studies. Most of an injected dose of radioiodide was concentrated in the skin and later released.

18. The iodide metabolism of the burbot is dominated by the skin, which may contain as much a 816 µg% of ^{127}I.

19. Seasonal changes in the stable iodide concentration of the serum parallel the change in water temperature during the year. Summer values of 30.8 µg% fall to 7.1 µg% in the winter.

20. The concentration of iodide in the skin shows a complex biphasic change. These changes appear to be partly determined by environmental temperature but other factors, as yet unknown, are involved. Skin iodide shows high concentrations in January (737 µg%) and October (816 µg%) with low values in May (334 µg%) and October (227 µg%).

21. The concentration of enzyme 1 shows changes during the year. Maximum activity occurs during the spring. Activities in the fall are high, whereas values for summer and winter are lower and approximately equal.

22. The relative concentrations of enzymes 2 and 3 show a marked reduction in activity during the fall. In the spring, as the lake temperature warms, the relative activity of these enzymes increases again.

BIBLIOGRAPHY

Acland, J.D. 1957. The estimation of serum protein-bound iodine by alkaline incineration. *Biochem.* 66:177-188.

Albright, E.C., R.W. Heninger and F.C. Larson. 1965. The effect of cold-induced hyperthyroidism on iodine-containing compounds of extrathyroid tissues in *Current Topics in Thyroid Research.* 346-355. C. Cassano and M. Andreoli ed. Acad. Press. New York.

Alpers, J.B., M.L. Petermann and J.E. Rall. 1956. Ultra-centrifugal studies of the process of thyroglobulin hydrolysis. *Arch. Biochem. Biophys.* 65:513-521.

Alpers, J.B. and J.E. Rall. 1957. The effect of thyrotropin on thyroglobulin hydrolysis. *Endocrinol.* 61:1:111-112.

Alpers, J.B., J. Robbins and J.E. Rall. 1955. The hydrolysis of rat thyroglobulin by thyroidal enzymes. *Endocrinol.* 56:1:110-119.

Anson, M.L. 1937. The estimation of cathepsin with hemoglobin and the partial purification of cathepsin. *J. Gen. Physiol.* 20:565-574.

Anson, M.L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* 22:79-90.

Baker-Cohen, K.F. 1959. Renal and other heterotopic thyroid tissue in fishes in *Comp. Endocrinol.* 283-301. A. Gorbman ed. J. Wiley and Sons Inc. New York.

Balasubramaniam, K. and W.P. Deiss, Jr. 1965. Characteristics of thyroid lysosomal cathepsin. *Biochem. Biophys. Acta.* 110:564-575.

Balasubramaniam, K., W.P. Deiss, Wee-Chong Tan and R.C. Powell. 1965. Effect of thyrotropin on iodoprotein of thyroid cell fractions. *Endocrinol.* 77:54-60.

Barker, S.B. 1964. Physiological activity of thyroid hormones and analogues in *The Thyroid Gland.* 1:199-236. R. Pitt-Rivers and W.R. Trotter, ed. Butterworths. London.

Barrington, E.J.W. 1957. The alimentary canal and digestion in *Physiology of Fishes.* 109-162. M.E. Brown, ed. Acad. Press. New York.

Baslow, M.H. and R.F. Nigrelli. 1964. The effect of thermal acclimation on brain cholinesterase activity of the Killifish, *Fundulus heteroclitus*. *Zoologica*. 49:2:41-51.

Berg, O., A. Gorbman and H. Kobayashi. 1959. The thyroid hormone in invertebrates and lower vertebrates *in Comp. Endocrinol*. 302-319. A. Gorbman, ed. J. Wiley and Sons Inc. New York.

Bodwell, C.E. and A.M. Pearson. 1964. The activity of partially purified bovine catheptic enzymes on various natural and synthetic substances. *J. Food Sci*. 29:5:602-607.

Bouma, J.M.W. and M. Gruber. 1964. The distribution of cathepsin B and C in rat tissues. *Biochem. Biophys. Acta*. 89:545-547.

Brandt, P.W. and A.R. Freeman. 1967. Plasma membranes substructural changes correlated with electrical resistance and pinocytosis. *Science*. 155:582-585.

Brown, M.E. 1957. Experimental studies on growth *in Physiology of Fishes*. Ch. 9. 361-400. M.E. Brown, ed. Acad. Press. New York.

Clements, M. 1957. Proteolysis by the thyroid and other tissues of the male and female dogfish, *Scyliorhinus (Scyllium) canicula*. *J. Exp. Zool*. 136:249-258.

Clements, M. and A. Gorbman. 1955. Protease in ammocoetes endostyle. *Biol. Bull*. 108:258-263.

Davis, H.S. 1956. *Culture and diseases of game fishes*. Univ. of California Press. Berkeley and Los Angeles.

Deiss, W.P. Jr., K. Balasubramaniam, R.L. Peake, J.A. Starrett and R.C. Powell. 1966. Stimulation of proteolysis in thyroid particles by thyrotropin. *Endocrinol*. 79:19-27.

DeRobertis, E. 1941. Proteolytic enzyme activity of colloid extracted from single follicles of rat thyroid. *Anat. Rec*. 80:219-231.

Dixon, M. and E.C. Webb. 1964. *Enzymes*. Longmans, Green and Co. Ltd. London. 950 pp.

Dopheide, T.A. and V.M. Trikojus. 1966. Hydrolysis of the A and B chains of oxidyzed insulin by thyroid acid proteinase. *Biochem. Biophys. Acta*. 118:435-437.

Ducommun, P. W. Vale, E. Sakiz and R. Guillemin. 1967. Reversal of the inhibition of TSH secretion due to acute stress. Endocrinol. 80:953-956.

Dziemian, A.J. 1943. Proteolytic activity of the thyroid gland. J. Cell. and Comp. Physiol. 21:3:339-345.

Eales, J.G. 1963. A comparative study of thyroid function in migrant juvenile salmon. Can. J. Zool. 41:811-824.

Eales, J.G. 1964. The influence of temperature on thyroid histology and radioiodine metabolism of yearling steelhead trout, Salmo gairdnerii. Can. J. Zool. 42:829-841.

Eales, J.G. 1965. Factors influencing seasonal changes in thyroid activity in juvenile steelhead trout, Salmo gairdnerii. Can. J. Zool. 43:719-729.

Ekholm, R. S. Smeds and U. Strandberg. 1966. Hydrolysis of thyroglobulin and B-glycerophosphate catalyzed by guinea pig thyroid particles. Exp. Cell. Research. 43:506-514.

Evans, R.M., F.C. Purdie and C.P. Hickman, Jr. 1962. The effect of temperature and photoperiod on the respiratory metabolism of rainbow trout (Salmo gairdnerii). Can. J. Zool. 40:108-118.

Evropeizeva, N.W. 1947. On the larval period of Lota lota L. (Teleostei Gadidae). Trav. Soc. Nat. St. Petersb. (Léning) 69:4:70-87. Eng. summary.

Field, J.B., S.M. Epstein, A.K. Remer and C. Boyle. 1966. Pyridine nucleotides in the thyroid. VI. DPN kinase activity of thyroid hemogenate obtained from slices incubated with and without thyroid stimulating hormone. Biochem. Biophys. Acta. 121:241-249.

Fontaine, M. and Y.A. Fontaine. 1957. Influence de l'origine zoologique des substances hypophysaires thyréotropes sur leur activité en fonction de la température. J. de Physiologie. 49:169-173.

Foss, O.P., L.V. Hankes and D.D. Van Slyke. 1960. A study of the alkaline ashing method for determination of protein-bound iodine in serum. Clinica Chemica Acta. 5:301-326.

Freeman, J.A. 1950. Oxygen consumption, brain metabolism and respiratory movements of goldfish during temperature acclimation, with special reference to lowered temperature. Biol Bull. 99:416-424.

Freinkel,N. 1964. The intermediary metabolism of thyroid tissue in The Thyroid Gland 1:131-162. R.Pitt-Rivers and W.R Trotter, ed. Butterworths. London.

Furlan,M. 1966. Specificity of intracellular acid proteinases for the hydrolysis of proteins. Enzymologia. 31:9-22.

Gennaro,J.F. and M.M.Clements. 1956. The incorporation of ^{131}I into frog skin in vitro and in vivo. Anat. Rec. 124:294.

Gomazkov,O.A. 1959. Effect of temperature of digestion rate of the burbot (Lota lota L) In Russian. Byull.Inst.Biol. Vodokhran 5:26-28 cited in H. Smith 1967. Influence of temperature on the rate of gastric juice secretion in the brown bullhead, Ictalurus nebulosus. Comp. Biochem. Physiol. 21:125-132.

Gorbman,A. 1957. Problems in the comparative morphology and physiology of the vertebrate thyroid gland in Comp. Endocrinol. 266-282 A.Gorbman, ed. J.Wiley and Sons Inc. New York.

Grainger, J.N.R. 1958. First stages in the adaptation of poikilotherms to temperature change in Physiological Adaptation. 79-91. C.L.Prosser, ed. Ronald Press. New York.

Grossman, A. and G. F. Grossman. 1955. Protein-bound iodine by alkaline incineration and a method for producing a stable cerate color. J.Clin. Endocrinol. and Metab. 15:3:354-361.

Haddad, H.M. and J.E.Rall. 1960. Purification of thyroid proteases by cellulose column chromatography. Endocrinol. 67:413-418.

Hale, L.J. 1958. Biological Laboratory Data. M. Abercrombie, ed. Methuen and Co. Ltd. London. 132 pp.

Hickman, C.P.Jr. 1959. The osmoregulatory role of the thyroid gland in the starry flounder, Platichthys stellatus. Can. J. Zool. 37:997-1060.

Hickman, C.P.Jr. 1962. Influence of environment on the metabolism of iodine in fish. Gen. and Comp. Endocrinol. Suppl. 1:48-62.

Hickman, C.P.Jr. 1967. Personal communication - unpublished data.

Hickman, C.P.Jr., R.A.McNabb, J.S.Nelson, E.D.Van Breemen and D.Comfort. 1964. Effect of cold acclimation on electrolyte distribution in rainbow trout (Salmo gairdnerii). Can. J. Zool. 42:577-597.

Hoar, W.S. 1957. The gonads and reproduction in Physiology of Fishes. Ch. 7:287-322. M.E. Brown, ed. Acad.Press. New York.

Hoar, W.S. and J.G. Eales. 1963. The thyroid gland and low-temperature resistance of goldfish. Can. J. Zool. 41:653-669.

Hoar, W.S. and G.B. Robertson. 1959. Temperature resistance of goldfish maintained under controlled photoperiods. Can. J. Zool. 37:419-428.

Hochachka, P.W. 1962. Thyroidal effects on pathways for carbohydrate metabolism in a teleost. Gen. Comp. Endocrinol. 2:499-505.

Hochachka, P.W. and F.R. Hayes. 1962. The effect of temperature acclimation on pathways of glucose metabolism in the trout. Can. J. Zool. 40: 261-270.

Hoffert, J.R. and P.O. Fromm. 1959. Estimation of thyroid secretion rate of rainbow trout using radioactive iodine. J. Cell. and Comp. Physiol. 54: 2:163-169.

Huang, C. 1967. A study of the binding of inorganic iodide to the plasma proteins of some freshwater teleost fishes. MSc Thesis U. of Alberta. Edmonton.

Hubbs, C.L. and K.F. Lagler. 1947. Fishes of the Great Lakes region. Cranbrook Inst. of Sci. Bull. #26.

Iodice, A.A., V. Leong and I.M. Weinstock. 1966. Separation of cathepsins A and D of skeletal muscle. Arch. Biochem. Biophys. 117:477-486.

Irie, M. 1962. The effect of thyrotropin on the hydrolysis of thyroglobulin. Endocrinol. 70:603-604.

Jablonski, P. and M.T. McQuillan. 1967. The distribution of proteolytic enzymes in the thyroid gland. Biochim. Biophys. Acta. 132:454-471.

Jacoby, G.H. and C.P. Hickman, Jr. 1966. Transformation reactions and spontaneous labelling of iodophenols during thin-layer chromatography of radioiodine ^{125}I. Can. J. Physiol and Pharmacol. 44:499-505.

Jankowsky, H.D. 1964. Die Bedeutung der Hormone für die Temperaturanpassung in normalen Temperaturbereich. Helgol. Wiss. Meeresunters. 9: 412-419.

Johnston, P.V. and B.I. Roots. 1964. Brain lipid fatty acids and temperature acclimation. Comp. Biochem. Physiol. 11:303-309.

Kamner, M.E., A. Peranio and M. Bruger. 1950. Factors affecting the measurement of proteolytic activity of thyroid tissue. *Endocrinol*. 46:353-358.

Klotz, I.M. 1950. The nature of some ion-protein complexes *in Coldspring Harbor Symposium on Quantitative Biology* 14:97-112.

Klotz, I.M. 1967. Protein subunits: A table. *Science* 155: 697-698.

Konishi, J. and C.P. Hickman, Jr. 1964. Temperature acclimation in the central nervous system of rainbow trout (*Salmo gairdnerii*). *Comp. Biochem. Biophys*. 13:433-442.

Kress, L.F., R.J. Peanasky and H.M. Klitgaard. 1966. Purification properties and specificity of hog thyroid proteinase. *Biochim. Biophys. Acta*. 113:375-389.

Kusakina, A.A. 1962. Relation of muscle and cholinesterase thermostability in certain fishes to specific environmental temperature conditions. *Tsitologiya* 4:1:68 transalted in *Fed. Proc*. Trans. Suppl. 22:1:123-126. 1963.

Langer, O. 1967. Personal communication. Unpublished data.

Lapresle, C. and T. Webb. 1962. The purification and properties of a proteolytic enzyme, rabbit cathepsin E, and further studies on rabbit cathepsin D. *Biochem. J*. 84:455-462.

Laver, W.G. and V.M. Trikojus. 1956. Complex nature of the proteolytic system of the thyroid gland. *Biochim. Biophys. Acta*. 20:444-445.

Lawler, G.H. 1963. The biology and taxonomy of the burbot, *Lota lota*, in Heming Lake, Manitoba. *J. Fish. Res. Bd. Can*. 20:2:417-433.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins *in Methods in Enzymology*. Vol. III 447-454. S.P. Colowick and N.O. Kaplan, ed. Acad. Press. New York.

Leloup, J. 1951. Fixation de radioiodide dans la thyroïde de deux sélaciens, *Scyllium canacula* L. et *Scyllium stellare*, Flem. *C.R.Acad. Sci*. 233: 635-637.

Leloup, J. and N. Fontaine. 1960. Iodine metabolism in lower vertebrates *in Modern Concepts in Thyroid Physiology*. *Annals of the New York Acad. of Science*. 86:2:316-353. O. Von St. Whitelock, ed.

Leloup, J. and F. Lachiver. 1955. Influence de la teneur en iode du régime sur la biosynthèse des hormones thyroïdienne. *C.R. Acad.des Sci*. 241:1: 509-511.

Lindsey, C.C. 1956. Distribution and taxonomy of fishes in the Mackenzie drainage of British Columbia. J. Fish. Res. Bd. Can. 13:6:759-789.

Litonjua, A.D. 1960. Proteolysis in phophate-purified thyroglobulin. Endocrinol. 67:829-838.

Lundblad, G., M. L. Bërnback and C. Widemann. 1960. Proteolytic activity in human thyroid extracts. 1. Purification and properties of some proteinases. Acta Chem. Scand. 20:675-688.

Loughlin, R.E., M.T. McQuillan and V.M. Trikojus. 1960. The influence of thyrotrophic hormone and methyl-thiouracil on the "cysteineyltyrosinase" (and proteinase) activity of the thyroid gland of the rat. Endocrinol. 66: 773-776.

Massey, V. 1953. Studies on fumarase. 31. The effect of temperature. Biochem. J. 72:72-79.

McNabb, R.A. 1963. Contribution of dietary and environmental iodide to iodine balance of rainbow trout (Salmo gairdnerii). MSc. Thesis. U. of Alberta. Edmonton.

McQuillan, M.T., J.D. Mathews and V.M. Trikojus. 1961. Proteolysis of thyroglobulin by thyroid enzymes. Nature. 192:333-336.

McQuillan, M.T., P.G. Stanley and V.M. Trikojus. 1954. A study of the action of purified protease on ^{131}I labelled thyroglobulin. Aust. J. Biol. Sci. 7:319-335.

McQuillan, M.T. and V.M. Trikojus. 1953. Purification and properties of thyroid protease. Aust. J. Biol. Sci. 6:617-629.

Menzies, C.A. and M.T. McQuillan. 1967. Partial purification and properties of a peptidase from thyroid glands. Biochim. Biophys. Acta. 132:444-453.

Nikolsky, G.V. 1963. The Ecology of Fishes. trans. from Russian by L. Birkett. Acad. Press. New York. 352 pp.

Olivereau, M. 1954. Hypophyse et glands thyroide chez les poissons. Étude histophysiologique de quelques corrélations endocriniennes, en particulier chez Salmo salar L. Ann. Inst. oceanogr. Monaco. 29:95-296.

Pastan, I. and S. Almqvist. 1966a. Hydrolysis of thyroglobulin by a mast cell enzyme present in rate thyroid homogenates. Endocrinol. 78:2:350-360.

Pastan, I., and S. Almqvist. 1966b. Localization of rat thyroid alkaline protease to mast cells. Endocrinol. 78:2: 361-366.

Peiss, C.N. and J. Field. 1950. The respiratory metabolism of excised tissues of warm and cold adapted fishes. *Biol. Bul*. 99:213-224.

Pickford, G.E. and J.W. Atz. 1957. The physiology of the pituitary gland of fishes. *New York Zool. Soc.* New York. 613 pp.

Poffenbarger, P.L., R.C. Powell and W.P. Deiss, Jr. 1963. *In vitro* thyrotropin-induced proteolysis in the thyroid gland. *J. Clin. Invest*. 42:2: 239-248.

Powell, R.C., M.A. Rahman and W.P. Deiss, Jr. 1964. Studies on the stimulation of thyroidal inorganic ^{127}Iodide release in vitro by thyrotropin. *Endocrinol*. 74:3:395-407.

Precht, H. 1958. Concepts of the temperature adaptation of unchanging reaction systems of cold-blooded animals *in* *Physiological Adaptation*. 50-78. C.L. Prosser, ed. Ronald Press Co. New York.

Precht, H. 1964. Uber die Resistenzadaptation wechselwarmer Tiere an extreme Temperaturen und ihre Ursachen. *Helgol. Wiss. Meeresunters*. 9:392-411.

Press, E.M., R.R. Porter and J. Cebra. 1960. The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. *Biochem. J*. 74: 501-514.

Prosser, C.L. 1962. Some temperature adaptations of poikilothermic vertebrates. Proc. Symposia on Arctic Biology and Medicine. 11. *Comp. Physiol. of Temp. Reg*. 1-41. J.P. Hannon and E.G. Viereck, ed. Arctic Aeromedical Lab. Fort Wainwright. Alaska.

Prosser, C.L. and F.A. Brown, Jr. 1961. *Comparative Animal Physiology*. W.B. Saunders Co. Philadelphia. 688 pp.

Reinwein, D. 1964. Hormonsynthesis und Enzymspektrum bei Erkrankungen der menschlichen Schilddruse. *Acta Endocrinologica,* Suppl. 94. 108 pp.

Reithel, F.J. 1963. The dissociation and association of protein structures. *Adv. in Protein Chemistry* 18:123-226. C.B. Anfinsen, Jr., M.L. Anson and J.T. Edsall, ed. Acad. Press. New York.

Rieck, A.F., J.A. Belli and M.E. Blaskovics. 1960. Oxygen consumption of whole animal and tissues in temperature acclimated amphibians. *Proc. Soc. Exp. Biol. Med*. 103:436-439.

Roberts, J.L. 1964. Metabolic responses of fresh-water sunfish to seasonal photoperiods and temperatures. *Helgol. Wiss. Meeresunters*. 9:459-573.

Ronald, K., H.C. Macnab, J.E. Stewart and B. Beaton. 1964. Blood properties of aquatic vertebrates. 1. Total blood volume of the Atlantic cod, Gadus morrhua L. Can. J. Zool. 42:1127-1132.

Rous, P. 1925. The relative reaction within living mammalian tissues 11. on the mobilization of acid material within cells, and the reaction as influenced by the cell state. J. Exp. Med. 41:399-411.

Schneider, A.B. and I.H. Goldberg. 1965. Effect of actinomycin on TSH-induced activities of hypophysectomized rat thyroid. Fed. Proc. 24:383.

Scott, T.W., S.M. Jay and N. Freinkel. 1966. Further studies on the action of pituitary thyrotropin on the individual phosphatides of thyroid tissue. Endocrinol. 79:3:591-600.

Shapland, C.G. 1964. Studies on a human thyroid protease. J. Med. Lab. Technol. 21:1-20.

Siegal, S. 1956. Nonparametric statistics for the behavioral sciences. McGraw Hill Book Co. Inc. Toronto.

Sizer, I.W. 1943. Effects of temperature on enzyme kinetics. Adv. in Enzymol. 3:35-62.

Snedecor, G.W. 1956. Statistical Methods. Iowa State College Press. Ames. Iowa. 534 pp.

Snoke, J.E. and H. Neurath. 1950. The proteolytic activity of striated rabbit muscle. J. Biol. Chem. 187:127-135.

Stoll, R., R. Maraud, J. Mounier and P. Blanquet. 1959. Sur le rôle des enzymes mucolytiques dans la protéolyse thyroidienne. C.R.Soc.Biol. 153:2015-2017.

Tata, J.R. 1964. Distribution and metabolism of thyroid hormones in The Thyroid Gland 1:163-186. R. Pitt-Rivers and W.R. Trotter, ed. Butterworths. London.

Taurog, A. and E.S. Evans. 1967. Extrathyroidal thyroxine formation in completely thyroidectomized rats. Endocrinol. 80:915-925.

Taurog, A. and D.T. Thio. 1966. TSH-induced thyroxine release from puromycin-blocked thyroid glands of intact rabbits. Endocrinol. 78:103-110.

Umana, R. 1967. Relationship between the proteolytic activity and the nitrogen content of the liver. Arch. Biochem. Biophys. 119:526-535.

Ushakov, B.P. 1964. Thermostability of cells and proteins of poikilotherms and its significance in speciation. *Physiol. Rev.* 44:518-560.

Vinogradov, A.P. 1953. *The elementary chemical composition of marine organisms*. Memoir of the Sears Foundation No. 2 Trans. from Russian by J. Efron and J.K. Setlow with bibliography ed. and enlarged by V.W. Odum. Sears Foundation for Marine Research, Yale Univ. New Haven.

Wallace, G.B. and B.B. Brodie. 1939. The distribution of iodide, thiocyanate, bromide and chloride in the central nervous system and spinal fluid. *J. Pharmacol. and Explt. Therapeutics*. 65:220-226.

Walter, C. and E. Frieden. 1963. The prevalence and significance of the product inhibition of enzymes in *Adv. in Enzymology* 25:167-274.

Watson, P. and V.M. Trikojus. 1957. Proteolytische Aktivitat der Rattenschilddruse: Wirkung von 2-thiouracil und thyreotropem Hormon. *Liebigs ann. Chem.* 607:215-218.

Wattiaux, R. and C. Deduve. 1956. Tissue fractionation studies 7. Release of bound hydrolases by means of Triton X-100. *Biochem. J.* 63:606-608.

Weiss, B. 1953. Peptidase and proteinase activity of beef thyroid tissue. *J. Biol. Chem.* 205:193-203.

Wikgren, B.J. 1953. Osmotic regulation in some aquatic animals with special reference to the influence of temperature. *Acta Zoologica Fennica* 71:1-102.

Wollman, S.H., S. Spicer and M.S. Burstone. 1964. Localization of esterase and acid phosphatase in granules and colloid droplets in rat thyroid epithelium. *J. Cell. Biol.* 21:2:191-201.

Woodhead, P.M.J. and A.D. Woodhead. 1959. The effects of low temperatures on the physiology and distribution of the cod, *Gadus morrhua* L., in the Barents Sea. *Proc. Zool. Soc. London*. 133:181-199.

Zenkevitch, L. 1963. *Biology of the seas of the USSR*. trans. by S. Botcharskaya. Lond. George Allen & Univin Ltd.

APPENDIX 1.

Studies on extraction of enzyme from fish thyroid tissue.

Table 1. Extraction of ^{125}I labelled thyroglobulin from sliced tissue by 0.9% NaCl for 12 hours. It was assumed that this would give an indication of the efficiency and consistency of extraction techniques. Dec. 11/64.

Weight g	Sex	Thyroid wt.mg	%^{125}I extracted	%^{125}I in wash	%^{125}I residue	Total ^{125}I
1056	F	215	88	4.6	7.2	99.8
1003	F	237	83	5.1	12.0	100.1
1199	F	232	87	6.5	6.2	99.7
2381	M	280	75	7.5	17.9	100.4
1223	M	216	88	-	11.6	100.0
1308	M	116	85	-	14.7	99.8

Table 2. Effects of detergents (Digitonin and Triton X-100) compared to basic saline extraction.

Treatment	Assay time min	pH	TU enzyme
Control	40	2.5	31.5
0.9% NaCl	80	2.5	57.8
Digitonin	40	2.5	29.5
2% in o.9% NaCl	80	2.5	57.0
Control	60	2.5	77.3
0.9% NaCl	60	3.25	78.1
	60	4.0	89.3
Triton X-100	60	2.5	71.5
% in 0.8%	60	3.25	70.8
NaCl	60	4.0	88.0

Table 3. Precision of enzyme analysis on hemoglobin substrate.

	mean TU	n	SD	$S\bar{x}$	range
pH 2.5 proteinase	99.41	12	1.71	0.492	97.0-102.6

APPENDIX 2.

Substrate preparation

Table 1 Effect on enzyme activity of substrate in suspension as opposed to substrate in solution.

Treatment	Concentration g%	Velocity TU/hr	Velocity after correcting for different substrate concentrations TU/hr 1.39g%	2.0 g%
Hemoglobin solution	1.39	45	45	47
Hemoglobin suspension	0.71	37	41	42

Table 2 Effect of substrate concentration on enzyme activity.

Substrate concentration g% hemoglobin	Enzyme Velocity 18/5/66 TU/hr	Enzyme Velocity 5/1/67 TU/hr
2.03	70	72
1.21	64	68
0.70	61	64
0.39	54	56
0.19	42	45
0.11	30	34

Table 3 Comparison of methods of hemoglobin assay.

Sample	Cyanmethemoglobin g% Hemoglobin	Phenol Reagent g% Hemoglobin	Gravimetric
A	5.36	2.25	7.41
B	2.90	1.85	5.12
C	--	1.43	4.34
D	--	0.93	2.21
E	--	0.52	1.13
F	--	0.28	0.59

APPENDIX 3.

Stable Iodide Analysis

Table 1. Brands of reagents found suitable for ^{127}I analysis.

1. Arsenious trioxide (As_2O_3)	Analytical reagent. Mallinckrodt Chemical Works
2. Brucine ($C_{23}H_{26}O_4N_2$)	Laboratory reagent. The British Drug Houses Ltd.
3. Ceric ammonium sulfate $(NH_4)_4Ce(SO_4).2H_2O$	Specially prepared for the protein bound iodine determination. The G. Frederick Smith Chemical Co.
4. Acid hydrochloric	Fisher reagent chemical Fisher Scientific Co. Ltd.
5. Sodium hydroxide	"Baker analyzed" reagent. J. J. Baker Chemical Co.
6. Potassium iodide	Reagent crystals Shawinigan McArthur Chemical Co. Ltd.
7. Sodium carbonate anhydrous	Reagent granular Shawinigan McArthur Chemical Co. Ltd.
8. Sulfuric acid	Reagent acid sulfuric Shawinigan McArthur Chemical Co. Ltd.
9. Zinc sulphate	Reagent granular Shawinigan McArthur Chemical Co. Ltd.

Table 2. Preparation of special solutions for ^{127}I analysis.

1. Arsenious acid ($HAsO_2$)	Stock solution. 3.8 g As_2O_3 dissolved in 50 ml warm N/1 NaOH. Add 50 m H_2O.
2. Arsenious acid solution (standards)	4 parts 3.5 N H_2SO_4 1 part $HAsO_2$
3. Arsenious acid (samples)	4 parts H_2O 4 parts 3.5 N H_2SO_4 1 part $HAsO_2$
4. Brucine	5 gm brucine / 1 liter 5% H_2SO_4
5. Calibration solution	5 parts H_2O 1 part 2 N HCl 1 part 4 N Na_2CO_3

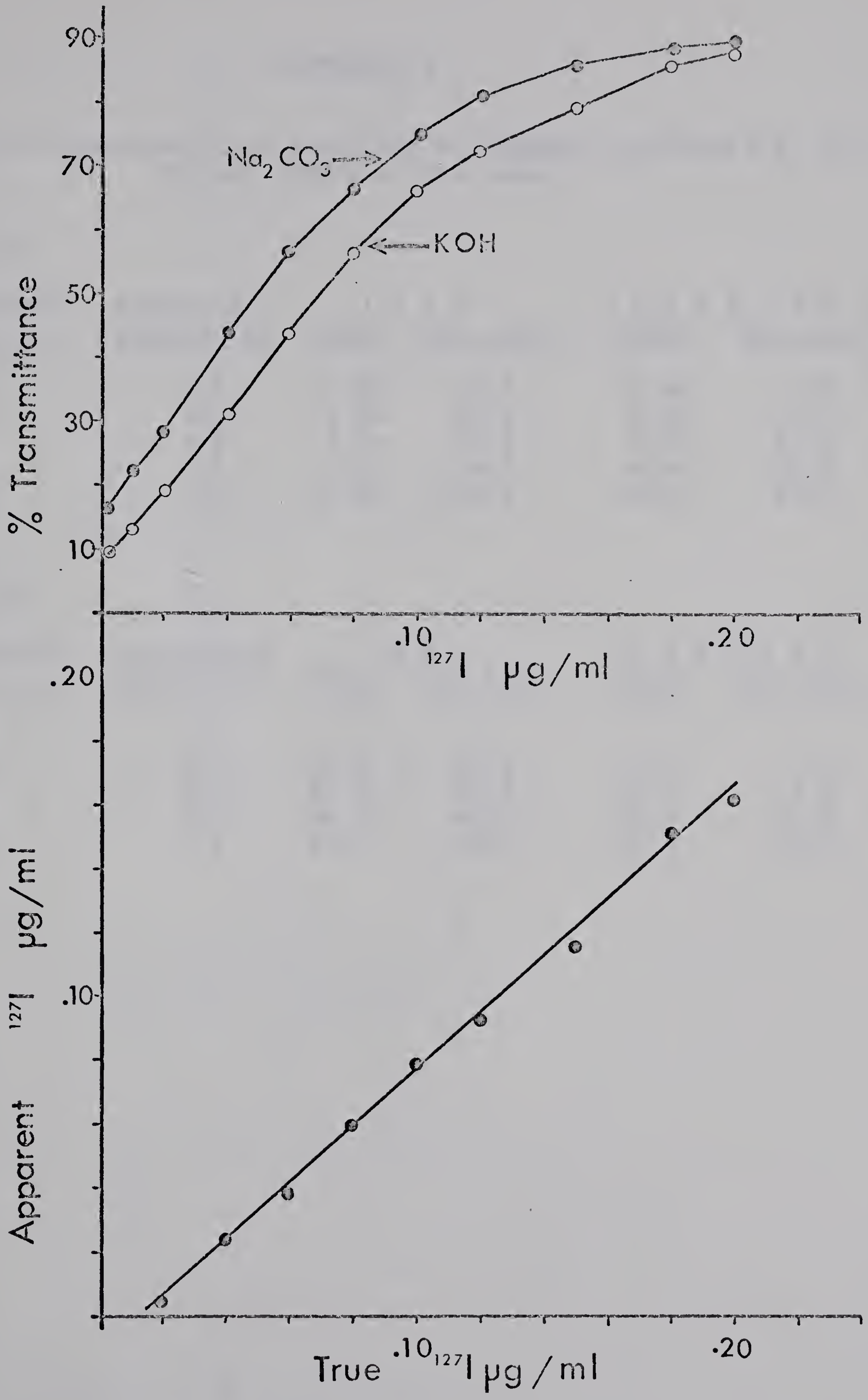
Table 3. Precision of Iodide analysis technique.

	mean	n	SD	$S_{\bar{x}}$	range
Serum ^{127}I	7.79 µg%	10	0.929	0.294	6.53-9.10µg%
Skin ^{127}I	3.24 µg/g	9	0.653	0.218	2.31-4.04µg/g

Table 4. Data showing effects of KOH on ^{127}I analyses.

Iodine conc. µ g/ml	% T Std. method	% T KOH method	Apparent iodine conc. KOH method ug/ml
H_2O	15.7	9.2	-
.01	21.9	12.8	-
.02	28.2	19.0	.006
.04	44.3	31.2	.02
.06	56.6	43.5	.079
.08	66.7	56.4	.060
.10	74.6	65.9	.079
.12	80.8	72.0	.093
.15	86.2	79.4	.116
.18	89.3	86.5	.153
.20	90.3	87.8	.165

T = transmittance


90
70
50
30
10
% Transmittance
Na$_2$CO$_3$
KOH
.10
.20
^{127}I µg/ml
.20
.10
Apparent ^{127}I µg/ml
True .10 ^{127}I µg / ml
.20

APPENDIX 4.

Effect of temperature on activity of enzymes collected at different times of the year.

Table 1A

Temperature C	Incubation period hr	JULY		FEBRUARY	
		TU/hr	Rel. Act.	TU/hr	Rel. Act.
0	9.6	1.96	17.4	2.40	7.8
5	6.0	2.89	25.8	3.61	11.7
10	4.0	4.10	36.5	5.50	17.9
15	3.0	6.12	54.5	9.95	32.3
20	1.5	11.23	100.0	18.47	60.0

Table 1B

Temperature C	Incubation period hr	MAY		JANUARY	
		TU/hr	Rel Act.	TU/hr	Rel Act.
0	-	-	-	-	-
5	5.0	18.0	21.2	6.5	8.3
10	2.8	25.6	30.2	11.4	14.5
15	2.0	40.8	48.0	18.4	23.4
20	1.0	68.0	80.0	31.5	40.0

APPENDIX 5.

Effect of TSH injection (2.5 IU in 0.5 ml.0.7% NaCl) on thyroid proteinase activity at pH 2.5 on hemoglobin substrate

Table 1	Date	Duration	Analytical Temp.	Temperature
	30/6/66	7 hr	30 C	15-16 C

Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU			Conus Enzyme TU		
				30 min	60 min	90 min	30 min	60 min	90 min
1610	F	23.2	Non injected Control	11.0	19.0	28.0	3.0	5.5	8.5
1265	M	20.0	Injected Control	8.5	17.5	27.5	1.0	3.0	6.5
1195	M	12.9	TSH	19.0	43.0	63.5	4.0	9.0	12.5
1300	F	21.0	TSH 7-8 hr	13.5	26.5	39.0	0.5	3.0	11.5
1340	F	16.1	Old, frozen TSH	5.5	15.5	26.0	5.0	8.5	9.0

Table 2	Date	Duration	Analytical Temp.	Temperature
	28/8/66	6-7 hr	30 C	12-15 C

Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU			Conus Enzyme TU		
				30 min	60 min	90 min	30 min	60 min	90 min
1635	F		6.3 hr Saline	28.5	47.0	65.5	11.0	20.0	28.0
935	M		6.5 hr Saline	21.5	39.0	51.0	13.5	24.5	33.5
1210	F		6.5 hr TSH	41.5	67.5	95.0	10.5	18.5	27.0
715	M		6.7 hr TSH	24.0	40.0	55.0	17.5	31.0	41.0

Table 3

Date	Duration	Analytical Temp.	Temperature
1/10/66	5-6 hr	30 C	14-15 C

Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU 30 min	Thyroid Enzyme TU 60 min	Thyroid Enzyme TU 90 min	Conus Enzyme TU 30 min	Conus Enzyme TU 60 min	Conus Enzyme TU 90 min
940	F	21.0	Control			80.5			39.0
1360	M	35.0	Control			66.0			32.5
1220	F	33.0	Control			76.0			28.5
1930	M	71.0	Control			70.0			55.0
1530	M	30.0	Control			82.0			45.5
						mean = 74.9			= 40.1
930	F	27.0	TSH			81.0			40.0
1510	M	43.0	TSH			91.0			38.5
1400	F	52.0	TSH			90.5			40.5
1700	F	83.0	TSH			78.0			37.5
760	M	28.0	TSH			69.5			27.7
						mean = 79.7			= 36.8

Table 4

Date	Duration	Analytical Temp.	Temperature
26/10/66	6 hr	30 C	14-16 C

Weight	Sex	Gonad	Notes	Thyroid Enzyme TU			Conus Enzyme TU		
g		wt g		30 min	60 min	90 min	30 min	60 min	90 min
1920	M	102.0	Non injected Control			131.0			
765	F	28.0	Non injected Control			78.5			
755	M	36.0	Non injected Control			58.0			
1450	M	53.0	Non injected Control			78.5			
						mean = 86.5			
1705	F	57.0	Inj. Control			86.0			
1755	F	72.0	Inj. Control			114.0			
1660	M	126.0	Inj. Control			73.5			
865	M	29.0	Inj. Control			81.0			
						mean = 89.1			
1125	M	60.0	TSH			48.5			
1705	F	204.0	TSH			78.0			
1505	F	109.0	TSH			188.5			
1535	F	69.0	TSH			85.0			
						mean = 100.0			

Effect of pituitary extract on thyroid proteinase activity at pH2.5 on hemoglobin substrate

Table 5	Date	Duration	Analytical Temp.	Temperature
	12/4/67	2 hr	25 C	4 C

Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU 30 min	Thyroid Enzyme TU 60 min	Thyroid Enzyme TU 90 min	Conus Enzyme TU 30 min	Conus Enzyme TU 60 min	Conus Enzyme TU 90 min
1190	M		Control			84.4			
1090	F		Control			75.7			
1050	F		Control			66.0			
					mean =	75.4			
1570	F		Exptl.			60.8			
940	M		Exptl.			57.0			
1070	F		Exptl.			77.0			
					mean =	64.9			

Effect of TSH injection (2.5 IU in 0.5 ml 0.7% NaCl) on Thyroid proteinase activity at pH 2.5 on hemoglobin substrate

Table 6		Date 13/10/66	Duration	Analytical Temp. 30 C			Temperature 14-15 C		
Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU 30 min	 60 min	 90 min	Conus Enzyme TU 30 min	 60 min	 90 min
1290	F		Saline-6 hr			24.5			8.0
933	M		TSH-6 hr			55.0			14.0
1315	M		Saline-12 hr			39.0			22.5
960	M		TSH-12 hr			56.0			18.3
1245	F		Saline-18 hr			54.3			25.5
1305	F		TSH-18 hr			61.5			17.0
945	F		Saline-24 hr			44.0			13.0
1427	M		TSH-24 hr			38.5			11.0

Effect of TSH injection (2.5 IU in 0.5 ml 0.7% NaCl) on thyroid activity measured on hemoglobin substrate

Table 7
Date 8/11/66
Analytical Temp. 30 C
Temperature 5 C

Weight g	Sex	Gonad wt g	Notes	Thyroid Enzyme TU 90 min			Conus Enzyme TU		
				pH 2.5	pH 3.25	pH 4.0	30 min	60 min	90 min
1385	M	155.0	Saline-1 hr	12.3	8.0	2.0			
1510	M	85.0	TSH-1 hr	15.0	8.5	2.5			
1598	M	265.0	Saline-2 hr	12.0	5.5	1.5			
1405	F	93.0	TSH-2 hr	14.0	7.5	5.0			
1680	M	230.0	Saline-4 hr	19.5	11.0	6.0			
1810	M	186.0	TSH-4 hr	16.5	3.0	2.0			
1250	M	178.0	Saline-6 hr	17.5	9.5	4.5			
1700	F	97.0	TSH-6 hr	12.0	8.0	3.0			
1820	M	147.0	Saline-12 hr	15.0	5.5	2.5			
1245	F	91.0	TSG-12 hr	13.0	6.0	2.5			
1155	M	168.0	Saline-24 hr	7.0	4.2	4.5			
1465	F	18.0	TSH-24 hr	7.0	1.5	2.5			
1620	M	149.0	Pit. extract 67 min	34.0	23.0	11.3			
1880	F	140.0	Pit. extract 70 min	24.1	17.0	11.0			
1810	F	108.0	Pit. extract 105 min	54.0	41.5	31.5			

APPENDIX 6.

Data from fish collected at different seasons

April 1964

Table 1

Weight	Gonad	GSI	Liver	LSI	^{127}I		Enzyme concentration (TU)			
g	wt.g		wt.g		µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy pH4.0	conus
Male										
1823	34	1.9			7.47					
947	108	11.4			5.83					
1611	24	1.5			6.77					
1333	17	1.3			9.57					
1429	35	2.5			7.70					
1452	20	1.4			10.50					
	mean =	3.3			= 7.97					
Female										
1225	30	2.5			4.90					
2081	63	3.0			4.43					
1752	49	2.8			10.27					
1897	38	2.0			3.97					
1781	62	3.5			7.70					
2142	72	3.4			5.13					
	mean =	2.9			= 6.07					

August 1964

Table 2

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1795	38	2.1			30.33					
1225	20	1.6			15.75					
1420	5	.4			30.91					
1327	4	.3			21.25					
	mean =	1.1			= 24.56					
Female										
1753	26	1.5			39.43					

May 17, 1966

Table 3

Weight	Gonad	GSI	Liver	LSI	^{127}I		Enzyme concentration (TU)			
g	wt.g		wt.g		µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1210	15	1.2	53	4.4	-	-	12.7	4.7	n.m.	14.6
2415	32	1.3	89	3.7	-	-	18.0	7.4	n.m.	15.4
1455	19	1.3	70	4.8	-	24.49	22.6	10.8	0.7	17.6
2284	41	1.8	71	3.1	12.25	27.08				
2059	26	1.3	111	5.4	16.45	50.37				
1782	26	1.5	72	4.0	-	57.37				
1948	27	1.4	84	4.3	12.01	52.73				
	mean =	1.4			= 13.57	= 42.41	= 17.8	= 7.6		=15.9
Female										
1795	40	2.2	87	4.8	-	-	36.0	21.1	3.8	15.4
1890	35	1.9	104	5.5	12.35	67.59	25.5	17.6	3.3	14.9
2035	50	2.5	97	4.8	11.78	62.48	15.6	7.9	1.1	5.5
1785	148	8.3	24	1.4	12.71	44.16				
	mean =	3.7			= 12.28	= 58.08	= 25.7	= 15.5	= 2.7	= 11.9

Table 4

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
2490	41	1.7	118	4.7	13.76	1.89	65.7	40.5	13.5	9.2
1620	35	2.2	96	5.9	18.20	3.79	57.1	31.4	13.4	11.0
1520	28	1.8	69	4.5	11.67	1.04	54.5	33.6	21.8	5.1
1690	13	.8	93	5.5	18.20	2.85				
1420	13	.9	88	6.2	15.40	5.35				
1500	14	.9	82	5.5	12.13	1.98				
1610	19	1.2	66	4.1	16.33	2.74				
1673	27	1.6	53	2.9	9.80	1.94				
1530	17	1.1	49	3.2	14.46	5.53				
960	14	1.5	73	7.6	-	-				
810	8	1.0	55	6.8						
	mean	= 1.3			= 14.44	= 3.01	= 59.1	= 35.2	= 16.2	= 8.4
Female										
2100	41	2.0	71	3.4	33.36	6.34	24.1	7.4	1.8	9.6
2070	21	1.0	147	7.1	12.50	3.69	43.5	20.6	5.9	9.7
1580	27	1.7	101	6.4	16.56	2.92	50.0	30.7	9.8	6.5
700	5	.7	58	8.3						
	mean	= 1.3			= 20.81	= 4.32	= 39.2	= 19.6	= 5.8	= 8.6

July 2, 1966

Table 5

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1640	19	1.2	80	4.9						
Female										
2030	45	2.2	38	1.9						
1296	19	1.5	23	1.7						
	mean =	1.9								

July 10 and 15, 1966

Table 6

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I µg% serum	^{127}I µg/g skin	Enzyme concentration (TU) thy. pH2.5	Enzyme concentration (TU) thy. pH3.25	Enzyme concentration (TU) thy. pH4.0	conus
Male										
1810	20	1.1	68	3.8						
1137	18	1.6	38	3.3						
1165	2	-	35	3.0						
2075	20	1.0	62	3.0						
875	9	1.0	19	2.2						
1575	42	2.6	20	1.3	19.13	5.61				
	mean =	1.5								
Female										
1250	15	1.2	32	2.5	-	-				
1100	12	1.1	58	5.3	35.69	2.48				
1620	28	1.7	34	2.1	37.56	2.40				
	mean =	1.3			= 36.63	= 2.44				

July 23, 1966

Table 7

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1550	17	1.1	43	2.8	12.83	1.99				
1145	6	-	53	4.6	13.41	5.11				
1215	10	-	39	3.2	24.96	3.69				
935	7	-	37	4.0	11.08	2.79				
				mean =	15.57 =	3.39				
Female										
1105	18	1.6	27	2.4	26.82	3.62				
1015	12	1.2	48	4.8	18.90	7.88				
	$\bar{u}$ =	1.4		=	22.86					

July 30, 1966

Table 8

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1695	37	2.2	50	2.9			16.1	9.9	2.4	8.9
1157	11	-	38	-			12.9	5.7	3.5	4.8
1283	7	-	49	-			14.6	6.7	2.1	5.4
						mean =	14.5	= 7.4	= 2.7	= 6.4
Female										
1905	28	1.5	90	4.7			8.8	2.2	0.4	2.8
1140	16	1.4	55	4.8			33.8	28.9	14.4	-
1170	20	1.7	63	5.4			-	-	-	9.9
1720	30	1.7	50	2.9			49.1	35.4	28.7	9.8
	mean =	1.6					= 30.6	= 22.2	= 14.5	= 7.5

September 2, 1966

Table 9

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1405	16	1.1	59	4.2			29.1	21.6	17.5	11.6
1385	14	1.0	64	4.6			33.7	26.1	11.7	7.9
2105	25	1.2	80	3.8			20.8	13.7	8.5	11.4
1850	20	1.1	90	4.9			25.7	15.2	10.6	5.9
1570	17	1.1	70	4.4			-	-	-	-
1195	14	1.2	44	3.7			-	-	-	-
1955	18	1.0	99	5.0			-	-	-	-
1890	22	1.2	62	3.3			-	-	-	-
	mean =	1.1					= 27.3	= 19.2	= 12.1	= 9.2
Female										
2085	50	2.4	65	3.1			47.4	29.7	19.7	8.8
1360	22	1.6	63	4.6			31.8	21.4	12.5	14.0
	mean =	2.0					= 39.6	= 25.6	= 16.1	= 11.4

September 30, 1966

Table 10

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			conus
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	
Male										
1054	-	-	-	-	30.33	3.86				
1296	-	-	-	-	37.33	4.93				
				mean =	33.83	= 4.39				
Female										
863	-	-	-	-	18.34	6.40				

October 7 and 9, 1966

Table 11

Weight	Gonad	GSI	Liver	LSI	^{127}I		Enzyme concentration (TU)			
g	wt.g		wt.g		µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
2080	106	5.1	88	4.2						
2332	92	4.0	135	5.8						
1325	39	3.0	52	4.0						
1662	63	3.8	67	4.0						
1860	48	2.7	41	2.2						
	mean =	3.7								
Female										
1400	63	4.5	68	4.9	20.53	99.47				
1035	38	3.7	79	7.6	12.36	63.56				
1865	86	4.6	81	4.3	-	-				
	mean =	4.3			= 16.45	= 81.52				

October 14 and 17, 1966

Table 12

Weight	Gonad	GSI	Liver	LSI	^{127}I		Enzyme concentration (TU)			
g	wt.g		wt.g		µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
2320	132	5.7	142	6.1	10.27	19.87	-	-	-	-
2438	130	5.3	106	4.4	6.30	25.35	57.0	-	-	-
2038	93	4.6	64	3.1	9.57	39.37	27.2	-	-	-
2318	146	6.3	105	4.5	11.90	31.33	26.0	-	-	-
	mean =	5.5			= 9.51	= 28.98	= 36.7			
Female										
1470	95	6.4	84	5.7	-	80.84	31.8	-	-	-
1630	89	5.5	104	6.4	-	20.58	24.9	-	-	-
1835	67	3.6	92	5.0	-	58.76	23.7	-	-	-
2000	102	5.1	134	6.7	-	46.30	-	-	-	-
	mean =	5.3				= 51.62	= 26.8			

October 29, 1966

Table 13

Weight	Gonad	GSI	Liver	LSI	^{127}I		Enzyme concentration (TU)			
g	wt.g		wt.g		µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1780	91	5.1	54	3.0	12.98	1.42	21.8	13.8	8.1	-
2445	29	9.0	75	3.1	10.12	1.83	40.5	25.9	17.5	14.3
	mean =	7.0			= 11.55	= 1.63	= 31.1	= 19.9	= 12.8	= 14.3
Female										
2257	72	3.2	75	3.3	9.56	2.25	27.2	15.1	9.9	5.3
2690	118	4.4	147	5.5	6.47	2.56	27.9	15.8	8.5	5.6
2035	105	5.2	153	7.5	8.91	2.47	34.5	20.1	8.4	9.6
2290	96	4.2	136	5.9	9.10	3.07	28.3	13.6	4.8	5.2
	mean =	4.3			= 8.51	= 2.59	= 29.5	= 16.2	= 7.9	= 6.4

January 5, 1967

Table 14

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
1900	447	23.5	76	4.0	35.23	4.63	12.4	1.1	0.5	3.4
1360	368	27.1	31	2.3	8.63	4.74	26.0	16.5	9.8	11.6
1640	429	26.2	38	2.3	13.58	5.07				
	mean =	25.6			= 19.14	= 4.81	= 19.2	= 8.8	= 5.1	= 7.5
Female										
1830	239	13.1	109	6.0	-	4.51	23.4	12.5	1.5	14.0
2200	219	10.0	77	3.5	7.16	3.06	32.9	18.4	4.1	7.7
1950	233	11.9	116	6.0	-	7.48	15.7	8.8	2.0	3.9
1310	136	10.4	86	6.5	9.17	5.10				
1620	217	13.4	123	7.6	5.11	5.88				
1480	208	14.1	120	8.1	3.03	3.56				
2290	247	10.8	148	6.5	32.20	2.30				
1790	253	14.1	124	6.9	7.47	3.84	17.0	5.7	2.1	7.6
970	89	9.1	66	6.8	9.54	3.86				
2420	290	12.0	157	6.5	17.31	2.95				
1370	144	10.5	75	5.5	9.57	3.38				
	mean =	11.7			= 11.17	= 4.17	= 22.2	= 11.3	= 2.4	= 8.3

January 28, 1967

Table 15

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I µg% serum	^{127}I µg/g skin	Enzyme concentration (TU) thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
2335	396	17.0	315	13.5	6.11	4.17	19.0			
Female										
2420	328	13.6	93	3.8	4.68	10.48	14.1			
1595	245	16.0	75	4.6	10.68	12.51	13.3			
1865	292	15.7	117	6.3	12.66	4.79	52.3			
1610	241	15.0	104	6.5	4.20	10.64	19.0			
2040	315	15.4	138	6.8	4.38	1.62	24.8			
	mean =	15.1			= 7.32	= 8.01	= 24.7			

February 28, 1967

Table 16

Weight g	Gonad wt.g	GSI	Liver wt.g	LSI	^{127}I		Enzyme concentration (TU)			
					µg% serum	µg/g skin	thy. pH2.5	thy. pH3.25	thy. pH4.0	conus
Male										
2810	573	20.6	70	2.5	8.12	-	9.6			
1730	405	23.4	72	4.2	10.28	-	15.6			
1965	373	19.0	61	3.1	9.19	-	24.5			
1710	396	23.2	57	3.3	13.65	-	-			
	mean =	21.5			= 10.34		= 14.9			
Female										
1375	241	17.5	55	4.0	8.70	-	-			
2945	522	17.7	77	2.6	8.75	-	8.1			
2180	498	22.8	77	3.5	13.69	-	27.3			
1720	350	20.3	54	3.1	7.47	-	41.4			
1880	340	18.1	129	6.9	7.56	-	-			
1460	-	-	57	3.9	8.91	-	-			
	mean =	19.3			= 9.18		= 25.6			

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